Probing lipoylation in *Plasmodium*

by

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Abstract

Malaria parasites, *Plasmodium spp.*, use the lipoate cofactor in two of its organelles: the apicoplast and the mitochondion. The apicoplast of the parasite uses an endogenous, biosynthetic pathway to lipoylate its apicoplast resident lipoate-dependent protein, the pyruvate dehydrogenase (PDH). Apicoplast lipoylation and the PDH are dispensible during the blood stage of the parasite, but have divergent phenotypes outside this stage. Lipoylation in the mitochondrion occurs through a scavenging pathway, and initial work suggests that mitochondrial lipoylation and at least one of the lipoylated substrates are essential for the blood-stage of the parasite.

In the first chapter, we probed the importance of protein lipoylation by expressing an exogenous gene from *Enterococcus faecalis* into various subcellular compartments in the parasite. This gene, dubbed lipoamidase, hydrolyzes the amide bond between lipoate and a lipoylated protein, effectively inactivating the substrate protein. This process reverses the reaction catalyzed by lipoate ligase, and serves as a proxy for knocking down or knocking out the lipoylation machinery in the parasite. Targeting lipoamidase to the apicoplast reduced, but did not eliminate, lipoylation in the apicoplast, with no obvious growth phenotype. This is consistent with multiple published experiments

that suggest apicoplast lipoylation is dispensable in the blood stage of the parasite. Targeting lipoamidase to the mitochondrion of the parasite resulted in a severe growth phenotype that was partially rescued by acetate supplementation. These data strongly suggests that mitochondrial protein lipoylation is essential for parasite survival.

We sought to expand upon this finding by systematically deleting the mitochondrial proteins that are lipoylated in malaria parasites. The first knockout experiments discussed are in the murine model, *Plasmodium berghei*. Initial attempts using conventional knockout constructs were unsuccessful, so we switched to constructs generated by Plasmogem in order to exploit their higher integration frequency. Knockout experiments resulted in successful knockout of the mitochondrial KDH E2 subunit with no obvious blood stage growth defect. The BCDH E2 locus is targetable using the Plasmogem constructs, but multiple and exotic techniques to try to single clone a knockout parasite failed, indicative of a severe growth phenotype. The putative shared mitochondrial E3 subunit was thought to be indispensible since this protein should be required for the function of all lipoylated proteins in the parasite mitochondrion. We found, however, that the E3 locus is targetable, but no single cloning attempt was made, so it is unclear whether the E3 deletion line would phenocopy the BCDH E2 knockout and display a severe growth phenotype. Finally, pilot lipoamidase experiments were performed in attempts to replicate the findings in the first chapter by targeting lipoamidase to the mitochondrion of *P. berghei*. We obtained genetic evidence of lipoamidase integration into the genome, and the inactive mutant was successfully cloned

and expressed lipoamidase. Continuation of these experiments in *P. berghei* may illuminate lipoylation phenotypes in the murine parasite.

Finally, attempts were made to disrupt the paralog KDH E2 and BCDH E2 subunits in *P. falciparum* using a single crossover strategy. The KDH was disruptible, but multiple attempts to single clone a parasite failed, suggesting facile plasmid excision at that locus. This may suggest that the KDH E2 knockout may have a subtle growth phenotype that prefers having an undisrupted KDH E2 locus. Multiple attempts to knockout the BCDH E2 locus failed, with two transfections resulting in what is likely spurious integration into a distal, dispensable locus. These results indicate that BCDH is essential for parasite growth. Since BCDH is thought to synthesize acetyl-CoA, the activity of BCDH may explain the finding that acetate supplementation is required to bypass the toxicity of lipoamidase.

All told, the sum of these experiments show that the *P. falciparum* and P. berghei exercise crucial, mitochondrial, lipoate-dependent biochemistry. This implicates the lipoate ligases, LipL1 and LipL2, as possible essential genes for the parasite. Knockout experiments suggest that the KDH is likely dispensible, but possibly useful, in parasite biochemistry, while the BCDH is important in generating acetyl-CoA for the parasite.

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Chapter 1

Lipoylation in *Plasmodium*

1.1 Introduction

Many aspects of central metabolism hinge on the lipoic acid cofactor. Recent work in *Plasmodium spp*. has revealed much regarding central metabolism, and the reliance on lipoic acid in these pathways. Malaria parasites have both a biosynthetic and salvaging pathway for lipoic acid, and many phenotypes have arisen based on production, acquisition and application of this cofactor. This review seeks to cover not only the complicated mechanisms of lipoylation, but also their affiliated pathways vis-a-vis the malaria parasite.

1.2 Lipoylation in the apicoplast of *P.falciparum*

Extensive biochemical work in yeast (Schonauer et al., 2009), bacteria (Ma et al., 2006; McLaughlin et al., 2016; Morris et al., 1995), *Arabidopsis* (Ewald et al., 2014) and mammals (Feng et al., 2009) has shed light on the mechanism in the ancient and highly difficult chemistry involved in lipoate biosynthesis (**Figure 1**). The apicoplast of *Plasmodium* appears to hold many of the same

biochemical players as these other organisms, so the following model of lipoate synthesis can be built on what has already been discovered.

First, the aliphatic backbone is built through fatty acid synthesis in the apicoplast. Fatty acids are thought to be built onto the apicoplast resident acyl carrier protein (ACP). Once the ACP is octanoylated, the octanoyl ligase, LipB, transfers the octanoyl group from the ACP to a specific lysine residue on the E2 subunit of the pyruvate dehydrogenase (PDH), resulting in an octanoylated PDH E2 subunit (Christensen and Cronan, 2010). Finally, LipA acts as a lipoyl synthase by using iron sulfur clusters generated by the apicoplast resident iron sulfur biosynthesis machinery to add the sulfurs onto positions C6 and C8, completing lipoate synthesis onto the PDH E2 subunit (**Figure 2**).

Work performed in the blood stage of *Plasmodium falciparum* suggests that this mechanism may hold true for the parasite. Rescue experiments in *E.coli* demonstrate that *Pf*LipB and *Pf*LipA can replace their *E.coli* homologs (Wrenger and Muller, 2004), suggesting that *Pf*LipB is an octanoyl transferase and *Pf*LipA is a lipoyl synthase. Biochemical work performed by our lab demonstrates that *Pf*LipA can indeed acquire iron sulfur clusters and is able to transfer iron sulfur clusters to substrates. Definitive confirmation of apicoplast specific expression of these proteins has yet to be performed; bioinformatics work suggests that *Pf*LipB is targeted to the apicoplast (Thomsen-Zieger et al., 2003), while a *Pf*LipA-leader peptide fused to GFP demonstrated localization outside of the mitochondrion (Wrenger and Muller, 2004). Though it is likely that both LipA and LipB are indeed expressed in the apicoplast, no definitive evidence yet exists to confirm this hypothesis.

1.3 Deficits in the apicoplast lipoylation model

1.3.1 LipB knockout does not abrogate apicoplast lipoylation

Disruption of LipB should prevent lipoylation of the PDH E2. However, disruption of *Pf*LipB results in reduced, and not eliminated, lipoylation signal as demonstrated through Western blotting against lipoylated proteins. This has been explained by the potential dual localization of one of the mitochondrial lipoyl ligases, LipL2, to both the apicoplast and the mitochondrion, resulting in partial lipoylation of the apicoplast PDH E2 by LipL2 (Gunther et al., 2007).

This hypothesis is somewhat problematic. LipL2 is a functional rescue of an *E. coli* LipB knockout as well as knockout of the *E.coli* lipoyl ligase knockout, suggesting that LipL2 can function both as an octanoyl transferase and a lipoyl tranferase (Gunther et al., 2007). However, *Pf*LipL2 rescue of the *E.coli* LipB knockout is quite poor, while rescue of *E.coli* lipoate ligase knockout is superior (Allary et al., 2007). Work with recombinant LipL2 protein demonstrates that LipL2 acts primarily as a lipoyl transferase (Afanador et al., 2017). These data suggest that though LipL2 may be able to function as an octanoyl transferase, it may not be sufficiently competent *in vivo* to perform this function.

Further experimentation is then required to clarify the mechanism of apicoplast lipoylation. A *Pf*LipB/LipL2 double knockout may confirm that both LipB and LipL2 can octanoylate the PDH E2, and confirms that LipL2 can be functionally localized to the apicoplast. Knockout of LipA and concomitant disappearance of PDH E2 lipoylation would confirm LipA's role in sulfuration of the octanoyl-PDH E2. Finally, a good amount of work needs to be

performed to determine what the source of the octanoate is for apicoplast lipoylation.

1.3.2 Unknown source of octanoate

The octanoate used to generate the carbon backbone of lipoate is thought to be built on the acyl carrier protein through fatty acid synthesis, and in *Plasmodium*, this pathway should be the apicoplast resident FASII pathway. However, it is unclear how fatty acid synthesis occurs in the parasite during the blood stage. Fatty acid synthesis canonically occurs by iterative elongation of malonyl-CoA generated by the acetyl-CoA carboxylase (ACC) using biotin as an essential cofactor. Though the ACC is expressed during the blood stage of the parasite life cycle, it is not biotinylated (Dellibovi-Ragheb et al., 2018), suggesting that this canonical pathway may not be active during the blood stage of the parasite life cycle. Metabolomic works appears to confuse this hypothesis, with parasites demonstrating nominal production of myristate as the only detectable glucose-derived, synthesized fatty acid (Botte et al., 2013), and palmitate and stearate synthesized from acetate (Yu et al., 2008). Parasites without FabI, the enzyme catalyzing the first step of fatty acid synthesis, appear capable of generating palmitate and stearate from acetate as well, suggesting a noncanonical fatty acid synthesis mechanism.

As such, it is unclear how the parasite can generate the octanoate needed to generate lipoate without fatty acid synthesis. The above-mentioned non-canonical fatty acid synthesis pathway, may be linked to lipoylation, as work in our lab demonstrates that FabI deficient parasites are still able to lipoylate

the PDH E2. However, it remains a formal possibility that octanoate is taken up from the host and ends up attached to the PDH E2. Ultimately, rigorous determination of how fatty acid metabolism occurs in blood-stage Plasmodium is required to generate hypothesis regarding apicoplast octanoylation of the PDH E2.

1.4 Lipoate-dependent biochemistry in the apicoplast of *Plasmodium falciparum*

1.4.1 Putative generation of acetyl-CoA by the PDH

The only substrate for lipoylation in the apicoplast is the E2 subunit of the apicoplast resident pyruvate dehydrogenase (PDH). A pyruvate dehydrogenase is canonically made up of three subunits: the E1, E2 and E3 subunits, and all three subunits are required for the conversion of pyruvate to acetyl-CoA. Biochemical work demonstrates that each subunit of the *Pf*PDH is catalytically competent, and all three subunits have been localized to the parasite apicoplast in the blood stage (Foth et al., 2004; Wrenger and Muller, 2004), suggesting that the apicoplast is a possible source of pyruvate-derived acetyl-CoA.

Knockouts of the *Pf*PDH E1 and E3 subunits are tolerated in the blood stage of the parasite, but result in inconsistent phenotypes. There was no observed growth phenotype in the E1 knockout. In addition, there appeared to be no significant change in glucose-derived acetyl-CoA, suggesting that the PDH may not be a major source of acetyl-CoA for the blood stage of the parasite (Cobbold et al., 2013). Knockout of the E3 subunit also resulted in no detriment to growth, but did result in highly synchronous parasite growth

(Laine et al., 2015).

The PDH E1 knockout is the only lipoate-involved genotype carried past the blood stage of *P.falciparum*. This knockout results in the inability to produce sporozoites from oocysts. This falls in line with the fatty acid synthesis knockouts. The shared phenotypes between these two pathway knockouts strongly implicate the need for a fully functional apicoplast PDH to generate precursors for fatty acid synthesis during the oocyst stage of parasite development. It would stand to reason that knockouts of *Pf*LipB and *Pf*LipA would share the same phenotype as those knockouts should inactivate the PDH. However, these experiments have yet to be performed.

1.4.2 Knowledge of all PDH biochemistry is incomplete

No knockout of the lipoylated PDH E2 subunit has been described in the literature. It would be interesting to observe the phenotype of the E2 knockout as it somehow remains lipoylated in the LipB knockout, making the LipB knockout an insufficient surrogate for the E2 knockout. It may also inform whether or not the PDH is engaged in canonical PDH activity, or if the different subunits participate in different biochemistries, especially considering the PDH E1's ability to decarboxylate both pyruvate and alpha-ketoglutarate (Chan, et al. 2013)

The apicoplast has been implicated in generating metabolites that may be responsible for growth or quorum sensing (Andrabi et al., 2018; Duvalsaint and Kyle, 2018; Emami et al., 2017). The highly synchronous growth exhibited by the E3 knockout may implicate the E3 as a member of this this metabolite

generation. Interestingly, knockout of *Pf*LipB resulted in faster development through the intraerythrocytic cycle (Gunther et al., 2007). These data may suggest that while the PDH may not be important for generation of acetyl-CoA during the blood stage, it may be using lipoate-dependent biochemistry to generate signaling molecules to mediate growth.

The ability to "knock out" the apicoplast (Yeh and Derisi, 2011) offers an intriguing means of further defining the phenotype of parasites without a complete apicoplast organelle. These lines could be assayed for growth kinetics and lipoylation to see if the loss of a fully intact organelle phenocopies the PDH E1 and/or the E3 knockout, and whether or not the PDH E2 can still be lipoylated. This would key into what biochemical pathways might still be functional in the degenerate apicoplasts.

1.5 Lipoylation in the mitochondrion of *Plasmodium falciparum*

Mitochondrial lipoylation occurs through a wholly different mechanism than the mechanism described in the apicoplast (Figure 2). Fully formed lipoate is scavenged from the host. Biochemical assays suggest that erythrocytes may be enriched for lipoic acid for use as a redox cycling agent (Constantinescu and Pick, 1995; Constantinescu et al., 1993), suggesting that red blood cells may be a reservoir of free lipoate for parasites. Parasites can take up free lipoate from erythrocytes and traffic them to the mitochondrion, where they can by attached to the three mitochondrial substrates: the E2 subunit of the branched chain alpha-ketoacid dehydrogenase (BCDH), the E2

subunit of the alpha-ketoglutarate dehydrogenase (KDH), and the H protein of the glycine cleavage system (GCV) (Allary et al., 2007).

Lipoate attachment occurs in a multi-step, redox gated fashion. First, lipoate is activated by the mitochondrially localized LipL1 (Wrenger and Muller, 2004) by consumption of ATP and adenylating free lipoate, forming lipoyl-AMP (LA-AMP). Under highly reducing conditions, LipL1's affinity for LA-AMP drops by around 100-fold, allowing for the previously mentioned apicoplast and mitochondrially localized lipoyl transferase, LipL2 (Gunther et al., 2007), to use LA-AMP to attach lipoate to two of the mitochondrial substrates: the E2 subunit of the BCDH or the E2 subunit of the KDH. Under less reducing conditions, LipL1 holds onto the activated LA-AMP, and can attach lipoate to its own substrate, the H protein (Afanador et al., 2014, 2017).

The redox environment of the mitochondrion of the parasite during the blood stage appears to be quite reductive (Mohring et al., 2017), suggesting that if this redox gating mechanism is true *in vivo*, the parasite preferentially lipoylates the mitochondrial BCDH and KDH E2 subunits over the H protein. Interestingly, Western blotting appears to show the BCDH and KDH subunits being lipoylated, while H protein lipoylation occasionally appears difficult to visualize, and was originally thought to be due to low abundance (Falkard et al., 2013), but this phenomenon may be at least partly due to this preferential lipoylation scheme.

Mitochondrial lipoylation is likely essential for the blood stage parasite. Treating parasites with lipoate analogs reduces mitochondrial lipoylation and results in inhibited parasite growth (Afanador et al., 2014; Allary et al., 2007).

A similar phenomenon was observed when an exogenous, bacterial probe, dubbed lipoamidase, delipoylated the mitochondrial proteins in *P.falciparum*, and induced a severe growth phenotype. Finally, LipL1 appears refractory to knockout (Gunther et al., 2009). Knockout of LipL1 would prevent lipoylation of all three lipoylated substrates in the mitochondrion, suggesting that at least one of the mitochondrial substrates is essential for survival in the blood stage of the parasite.

The importance of mitochondrial lipoylation in the blood stage of the parasite will likely make normal knockouts experiments difficult, if not impossible, which would also obviate the ability to interrogate the importance of mitochondrial lipoylation outside the blood stage. As such, alternative means to alter protein expression, such as the auxin induced degron (Kreidenweiss et al., 2013), Flp/FRT recombination (Lacroix et al., 2011; van Schaijk et al., 2010), TetR DOZI (Ganesan et al., 2016), or the knock-sideways method (Birnbaum et al., 2017), may be required to more fully address the question of essentiality of mitochondrial lipoylation.

1.6 Lipoate-mediated metabolism in the mitochondrion of *Plasmodium falciparum*

1.6.1 Mitochondrially generated acetyl-CoA may be crucial for blood-stage parasite survival

Expression of active lipoamidase in the mitochondrion of *P.falciparum* resulted in significant reduction in lipoylation of the mitochondrial substrates,

along with a significant growth defect. This would suggest that lipoate-dependent metabolism in the parasite mitochondrion is important for blood stage survival. This growth defect is partially rescuable by acetate supplementation. Acetate can be taken up by the parasite and readily converted into acetyl-CoA by the cytosolic acetyl-CoA synthetase (Cobbold et al., 2016); this would argue that mitochondrial lipoylation is required for mitochondrially generated acetyl-CoA generation that is crucial for parasite growth.

The BCDH is one candidate that may be responsible for generating acetyl-CoA. Like the apicoplast resident PDH, the BCDH is composed of an E1, E2 and E3 subunit. All three subunits have been localized to the mitochondrion of the parasite (Afanador et al., 2014; Gunther et al., 2005). The E3 subunit, the dihydrolipoamide dehydrogenase, is likely shared between the three lipoate-dependent enzyme complexes in the mitochondrion as there appears to be only one encoded in the parasite genome (Laine et al., 2015). BCDH's are canonically associated with the catabolism of branched chain amino acids, i.e. leucine, isoleucine and valine. However, the parasite does not appear to code for enzymes upstream of the BCDH that are involved in the catabolism of branched chain amino acids, nor does the parasite appear to catabolize branched chain amino acids in culture (Cobbold et al., 2013). The parasite does appear to have a functional PDH-like enzyme that is not the apicoplast PDH, as the parasite appears able to convert glucose-derived pyruvate to acetyl-CoA independent of the apicoplast PDH (Cobbold et al., 2013). If this is correct, the BCDH may be an essential enzyme that cannot be disrupted.

The other candidate that may be generating acetyl-CoA in the mitochondrion is the KDH. All members of the KDH have been localized to the mitochondrion (Afanador et al., 2014; Chan et al., 2013), where it is canonically a member of the citric acid cycle, converting alpha-ketoglutarate to succinyl-CoA. Genetic, metabolomics, and biochemical work have confirmed this canonical activity (Chan et al., 2013; Ke et al., 2015). However, *in vitro* work with the E1 subunit of the KDH demonstrated the ability to use both alpha-ketoglutarate as well as pyruvate as reasonably equivalent rates (Chan et al., 2013).

If this is the case *in vivo*, both the BCDH and the KDH may be competent at converting pyruvate to acetyl-CoA. This has been partially demonstrated by functional complementation by *Pf*BCDH E1 of a *Pb*BCDH E1 knockout that rescues a growth phenotype tied to the generation of acetyl-CoA (Oppenheim et al., 2014). Biochemical work with the BCDH may help clarify what the most likely substrate is for the BCDH, while combinatorial knockouts of the BCDH and KDH may demonstrate if either or both are responsible for acetyl-CoA generation in *P.falciparum*. The acetate rescue observed in the lipoamidase experiments may provide rescue for possible lethality of these knockouts by exploiting the cytosolic acetyl-CoA- synthetase to provide an alternative means to generate acetyl-CoA.

1.6.2 Glutamine metabolism during the blood-stage and beyond

Knockout of the KDH E1 subunit results in no significant growth phenotype during the blood stage of parasite development, revealing the dispensability of the conversion of alpha-ketoglutarate to succinyl-CoA. This is an interesting finding, considering the parasite's auxotrophy for glutamine (Liu et al., 2006) and preference for glutamine catabolism for fueling the citric acid cycle (MacRae et al., 2013). This would suggest a robust compensatory mechanism for carbon metabolism (Ke et al., 2015), and the possibility that glutamine auxotrophy during the blood stage is primarily for protein synthesis. Outside the blood stage, the KDH E1 knockout does not produce viable oocysts.

It is interesting that disruption of several, closely metabolic pathways, including the citric acid cycle, apicoplast PDH function (Cobbold et al., 2013), fatty acid synthesis (van Schaijk et al., 2014), and heme metabolism (Ke et al., 2014) converge in mosquito stage arrest. This would argue that the mosquito stage of the parasite is a shift from a more auxotrophic mode in the blood stage to a more autotrophic mode.

1.7 Lipoylation in the apicoplast and mitochondrion of *Plasmodium berghei*

The use of murine models allows for interrogation of the complete malaria life cycle. However, the panoply of work performed in the interrogation of lipoylation and lipoate-dependent catalysis reveals significantly different biochemistry between the different malaria species. Part of this different

biochemistry appears to be two lipoylation enzymes that appear to have activity in both the apicoplast and the mitochondrion of the parasite, a finding different from what is observed in *P.falciparum*.

1.7.1 LipB

The *Plasmodium berghei* octanoyl transferase ortholog (LipB) is thought to be localized to the apicoplast, but this has not been confirmed, nor has its biochemical activity been confirmed. Knockout of *Pb*LipB in the blood stage results in reduced lipoylation of the apicoplast resident PDH E2 subunit, similar to findings in *P.falciparum* (Falkard et al., 2013). However, the *Pb*LipB knockout also results in complete abrogation of lipoylation signal of the mitochondrially residing BCDH E2 subunit, suggesting a role for LipB in both apicoplast and mitochondrial lipoylation, including the potential for dual localization of *Pb*LipB, an observation different from the findings of the *P.falciparum* homolog, and an interesting parallel to the dual localization of the *Pf* LipL2.

The growth phenotype associated with the *Pb*LipB knockout are also different from *P.falciparum* LipB knockout. While the *Pf* LipB knockout results in faster erythrocytic growth (Gunther et al., 2007), the *Pb*LipB knockout results in slower erythrocytic growth only under serum lipid depleted conditions, suggesting some involvement of *Pb*LipB in lipid metabolism that may not be the case in *P.falciparum*. *Pb*LipB parasites appear more susceptible to lipoate analog treatment when compared to wild type parasites, but whether or not this is due to reduced lipoylation of the PDH E2, BCDH E2 or both is unclear.

The *Pb*LipB knockout is able to progress through mosquito infection and eventually develop in the liver, where the lipoylation in the apicoplast is significantly decreased, confirming *Pb*LipB's role in lipoylation in the apicoplast (Falkard et al., 2013). FASII knockouts also show minimal lipoylation of the apicoplast during this stage (Deschermeier et al., 2012), suggesting that *P.berghei*, at least in the liver stage, uses synthesized fatty acids to synthesize lipoate in the apicoplast. Ultimately, parasites deficient in LipB are less infective, but still able to progress to the blood stage (Nagel et al., 2013), suggesting that there is sufficient redundancy in lipoate-mediated biochemistry between the apicoplast and the mitochondrion to progress throughout the life cycle.

1.7.2 LipL1

Initial attempts to knockout LipL1 in *P.berghei* were sublethal (Gunther et al., 2009), suggestive of the essential nature of LipL1 for mitochondrial lipoylation. Using a conditional knockdown system, reduced expression of LipL1 imparts a growth defect with concomitant reduced lipoylation of the BCDH and KDH E2 subunits, respectively (Wang et al., 2017). Surprisingly, LipL1 knockdown also resulted in reduced lipoylation of the apicoplast resident PDH E2 subunit. In another twist, knockdown of LipL1 appears to result in eventual compensatory upregulation of LipL2 that is concomitant with restoration of lipoylation of the BCDH, KDH and PDH E2 subunits and normal parasite growth (Wang et al., 2017).

This data would suggest that both *Pb*LipL1 and *Pb*LipL1 are somehow

responsible for lipoylating the apicoplast resident PDH E2 and the mitochondrion resident BCDH E2, a paradigm that appears distinct from *P.falciparum*. Considering the lack of clarity in the *P.falciparum* model, even more work is required to clarify the different paradigm in rodent models.

1.8 Lipoate-dependent metabolism in the apicoplast of murine models of malaria

The PDH E1 and E3 subunits were localized to the apicoplast in *Plasmodium yoelii* during the blood, sporozoite and intrahepatic stages of parasite development. Knockouts of either result in no detriment in blood or mosquito stages, but arrest at the late liver stage (Pei et al., 2010). Liver stage arrest also occurs in fatty acid synthesis knockouts (Vaughan et al., 2009), implicating the need for a fully functional FASII pathway that may rely on PDH activity for generation of acetyl-CoA during the liver stage of *P.yoelii*.

A slight variation on this theme occurs in *P.berghei*. The PDH E1 knockout in *P.berghei*, has no mentioned blood or mosquito stage phenotype, but a severe delay to patency in the liver-to-blood transition (Nagel et al., 2013). As in *P.falciparum* and *P.yoelii*, it appears that FASII and apicoplast resident, lipoate-mediated metabolism converge, as the LipB knockout (Falkard et al., 2013), FASII knockouts (Yu et al., 2008), PDH E1 knockout (Nagel et al., 2013), and the biotin ligase knockout (Dellibovi-Ragheb et al., 2018) all result in a severe, late-liver stage phenotype, but can still progress to blood stage infection. This demonstrates the importance, but not essentiality, of a fully functional repertoire of fatty acid synthesis players required for parasite development in

the liver stage.

It should be noted that the different species of malaria described have fatty acid synthesis and lipoate-mediated metabolism all converge within a species, but not between species. As mentioned above, most metabolic pathways seem to converge in importance in the mosquito stage of *P.falciparum*, while *P.berghei* and *P.yoelli* demonstrate reliance on similar pathways during the liver stage of infection. This highlights crucial, metabolic differences between the different species of malaria that need to be considered when drawing comparisons between them.

1.9 Lipoate-dependent metabolism in the mitochondrion of *P.berghei*

It is unclear whether or not lipoylation of the BCDH E2 is required for blood stage growth. As in *P.falciparum*, the *Pb*BCDH lipoylated E2 subunit has been localized to the mitochondrion of the blood stage parasite, but unlike *P.falciparum*, it is lipoylated both by either LipB (Falkard et al., 2013) or LipL1/LipL2 (Wang et al., 2017). Genetic work on these enzymes suggest that lipoylation of the BCDH may not be important to blood stage development. However, knockout of the E1 subunit imparts a severe, blood stage growth phenotype that identifies the *Pb*BCDH E1 as a functional pyruvate dehydrogenase E1 subunit (Oppenheim et al., 2014).

It is unclear how the *Pb*BCDH complex requires an E1 subunit, but can tolerate an unlipoylated BCDH E2 subunit to perform this biochemistry. Knockout of the BCDH E2 subunit in Chapter 3 may phenocopy the BCDH E1

knockout, as multiple attempts to single clone the knockout failed. This would suggest that the BCDH E1 an E2 subunits are crucial for generation of acetyl-CoA, but can perform this biochemistry without a lipoyl moiety. More rigorous interrogation of the BCDH needs to be performed to resolve this problem.

Knockout of the BCDH E1 subunit results in arrest in the oocyst stage. This finding is in contrast to the somewhat shared phenotype in *P.berghei* of liver stage arrest in FASII, PDH, and biotin ligase knockouts. Knockout of the ATP synthase in *P.berghei* appears to impart little effect on male gametocytes, but inhibits development of female gametes, resulting in arrest prior to oocyst development (Sturm et al., 2015). These data may suggest that mitochondrial lipoate-dependent biochemistry is important in mosquito stage development, while apicoplast lipoate-dependent biochemistry is important in liver stage development for murine parasites.

Some work has been done on the other mitochondrial lipoate-dependent proteins in murine models. The *Pb*KDH E2 subunit was knocked out in Chapter 3 with no gross phenotype observed, obviating the KDH as a candidate for imparting the growth phenotype observed in the *Pb*LipL1 knockdown (Wang et al., 2017). Attempts to knockout the *P.berghei* H protein in our lab and by others have failed (Varadarajan et al., 2014), implicating the H protein as an essential protein, but for unknown reasons.

1.10 Potential non-canonic lipoate-dependent biochemistry in *Plasmodium spp.*

The H protein is in the mitochondrion of *P.falciparum* (Spalding et al., 2010), but its role is unclear. H proteins are canonically associated with the multi-enzyme glycine cleavage complex (GCV) that is involved in the metabolism of glycine, serine and folate. GCV's are typically composed of 4 proteins: the P, T, L and H proteins. *P.falciparum* and *P.berghei* appears to code only for a T and H protein, and neither appear to perform GCV affiliated glycine catabolism during the blood stage (Varadarajan et al., 2014). It is appealing to consider the essentiality of the H protein considering the dual lipoylation system at play - why not just have a single lipoyl ligase, and why have it be redox sensitive?

Redox-dependent activity is not unique to mitochondrial lipoylation in *P.falciparum*. The parasite's essential, cytosolic serine hydroxymethyltransferase (SHMT) (Pornthanakasem et al., 2012) is also redox sensitive (Chitnumsub et al., 2014), with a crucial disulfide bond that needs to be broken in order to bind its substrate and perform catalysis. In the apicoplast, the acyl carrier protein also appears redox sensitive, with the ability to form dimers *in vitro* with exposed sulfhydrals from its phosphopantotheinate cofactor, but stays as monomers *in vivo* (Gallagher and Prigge, 2010). The parasitophorous vacuole has its own redox sensitive enzyme, SUB1, a serine protease that has a partially solvent exposed disulfide bridge that can mediate activity (Withers-Martinez et al., 2014). As such, there has been a redox-switchable enzyme described in nearly all compartments of the parasite.

Considering the variety of hosts and their respective oxidative environments, the parasite may have a redox sensitive program to react to these different environments. In the case of the mitochondrion, the normal condition of having a highly reductive environment (Mohring et al., 2017) may push the lipoylation balance to favor lipoylation of the BCDH and KDH to favor canonical metabolic roles, while oxidative insult results in sacrificing this metabolism for whatever function is involved in the H protein. Indeed, it is attractive to think of possible redox-sensitive programs within parasite organelles, or even cooperatively throughout the entire parasite.

Redox flux mediated through lipoylated proteins has been described elsewhere in life. E3 subunits (dihydrolipoamide dehydrogenases) have been implicated as a redox sink/sensor in bacteria (Bryk et al., 2002; Feeney et al., 2011; Gonidakis et al., 2010), parasites (Nyvltova et al., 2016) and human cells (Glasser et al., 2017; McLain et al., 2011), suggesting lipoate-dependent proteins regularly participate in complex redox flux. These demonstrate that a protein with a lipoyl domain and a cognate dihydrolipoamide dehydrogenase can work independently of decarboxylation chemistry to flux redox balance through lipoate's reactive thiols. This has demonstrated biochemically, where E3 subunits from several forms of life, including those of *P.falciparum*, can catalyze both the oxidation and reduction of lipoate's thiols at reasonably comparable speeds (Hakansson and Smith, 2007; Laine et al., 2015). It is possible that in addition to any canonical metabolic roles of the lipoylated proteins in *Plasmodium*, they can also act as a redox sinks/sensors in the parasite. This possibility is underscored by initial screens detecting substantive nitrosylated

or glutationylated *Plasmodium* proteins involved in lipoate-mediated catalysis (Kehr et al., 2011; Wang et al., 2014) .

It has been recently demonstrated that *Staphyloccocus aureus* can secrete its lipoylated PDH E2 subunit to suppress TLR-mediated macrophage activation, allowing for superior survival in mice against mutants that cannot lipoylate the PDH E2 subunit (Grayczyk et al., 2017). The ability for humans to immunologically recognize a lipoyl motif has already been demonstrated, as autoreactive antibodies are generated to the lipoyl domain of the human PDH E2, indicating a precedence for immune recognition (Pacini et al., 2015). Moonlighting of canonical proteins, including those involved with lipoate, as virulence factors and immunological phenomena has been described in other pathogens (A Ala et al., 1996; Hallstrom et al., 2015; Henderson and Martin, 2011), but some proximal examples in *Plasmodium* are beginning to appear, including surface expressed GAPDH (Cha et al., 2016) and enolase (Vora et al., 2009), as well as the growing understanding of the parasite exosomal effects on the host (Mantel and Marti, 2014), opening the door to noncanonical secretion or surface targeting of factors playing a role in parasite growth or pathogenesis. Considering these observations, the application of genetic work on the parasite in the mouse and mosquito should be applied in order to look for non-biochemical phenotypes to highlight immunological or other phenotypes, or may help to explain contradictory phenotypes between species.

1.11 Conclusion

Lipoylation is an ancient, ubiqtuitous and difficult chemical reaction, inextricably tied to central and peripheral metabolism. The role of lipoylation in the apicoplast of *Plasmodium* has not been fully determined, since no means of complete abrogation of apicoplast lipoylation has yet been described. However, shared phenotypes with fatty acid synthesis knockouts within mouse species and human species are suggestive of a general shift to a more autotrophic mode in the liver and mosquito hosts, respectively. Mitochondrial lipoylation appears to be a simpler story, with parasite auxotrophy for lipoylation demonstrated in the blood stage, and two downstream enzymes demonstrating essentiality after the blood stage.

Though much work has been performed in investigating *Plasmodium* lipoylation, it appears that the field has generated more questions than answers. Knockouts at different points in lipoylation and in different parasite species result in different, and possibly contradictory, phenotypes. Biochemical inferences have been made for the outcomes of these knockouts, but comparative genetic and metabolomics work indicate that there are major differences in the biochemistries between the different *Plasmodium* species, suggesting that assumed similarities between the species may be erroneous. This is further complicated by host difference, such as the existence of reticulocytes in mice that can provide metabolite rich reservoirs (Srivastava et al., 2015) that may mask auxotrophy observed in tissue culture of *P. falciparum*. Noncanonical roles of these proteins must be considered in evaluating the functions of the lipoylation machinery and their respective substrates, especially in the context

of the H protein, which necessitates assay of both the human and murine species of *Plasmodium* to consider each models different biochemical and immunological contexts.

Exploiting the necessity of *Plasmodium's* blood-stage lipoate scavenging indicates that drug intervention in this pathway is feasible. Growth inhibition in blood-stage parasites by lipoate analogs demonstrates the proof of principle in *in vitro* or *ex vivo* conditions (Afanador et al., 2017; Falkard et al., 2013). Humans and mice are dependent on endogenous, biosynthetic lipoylation, with lipoate supplementation unable to rescue genetic defects abrogating lipoylation (Mayr et al., 2014), indicative of an absent or inefficient scavenging pathway, suggesting that *in vivo* intervention may be possible. Indeed, several lipoate derivatives have been tested to some success in several disease models (Nakano et al., 2017; Yang et al., 2016; Zhou et al., 2017). One lipoate derivative, CPI-613, is undergoing multiple clinical trials due to its ability to dysregulate PDH and KDH activity in several cancer models (Alistar et al., 2017; Lamar et al., 2016; Pardee et al., 2014). The growing interested in exploiting lipoate derivatives can be capitalized in *Plasmodium* research to both find new means for malaria treatment and to concretize why lipoate is important to the parasite.

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1.13 Tables

Gene	Species	Blood Stage Phenotype	Other
LipB	*P. falciparum* (Günther et al., 2007)	Slower growth kinetics Reduced PDH lipoylation	
	P. berghei (Falkard et al., 2013)	Slower growth kinetics only under lipid depletion Reduced PDH and BCDH lipoylation	Delayed liver stage development
Apicoplast PDH	*P. falciparum (E1)* (Cobbold et al., 2013)	None	Oocyst arrest
	P. yoelli (E1, E3) (Pei et al., 2010)	None	Liver stage arrest
	P. falciparum (E3) (Laine et al., 2015)	Synchronous growth	
	P. berghei (E1) (Deschermeier et al., 2012; Nagel et al., 2013)	None described	Delayed liver stage development
LipL1	*P. falciparum* (Günther et al., 2009)	Unsuccessful	
	P. berghei (Günther et al., 2009)	Disruptible	
	P. berghei (knockdown) (Wang et al., 2017)	Initial growth defect rescued by LipL2 overexpression	
KDH	*P. falciparum (E1)* (Ke et al., 2015)	None	Oocyst arrest
	P. berghei (E2)	None observed	
	P.falciparum (E2)	Disruptible	
BCDH	*P. berghei (E1)* (Oppenheim et al., 2014)	Severe growth defect, partially rescued by acetate	Oocyst arrest
	P. berghei (E2)	Disruptible	
	P.falciparum (E2)	Unsuccessful	
H protein	*P. berghei* (Varadarajan et al., 2014)	Unsuccessful	

Table 1.1: Summary of knockouts performed on *Plasmodium* lipoylation machinery and the substrates. Knockout attempts of the *Pb/Pf* KDH E2, *Pb/Pf* BCDH E2 and *Pb*H protein were performed by our lab.

1.14 Figures

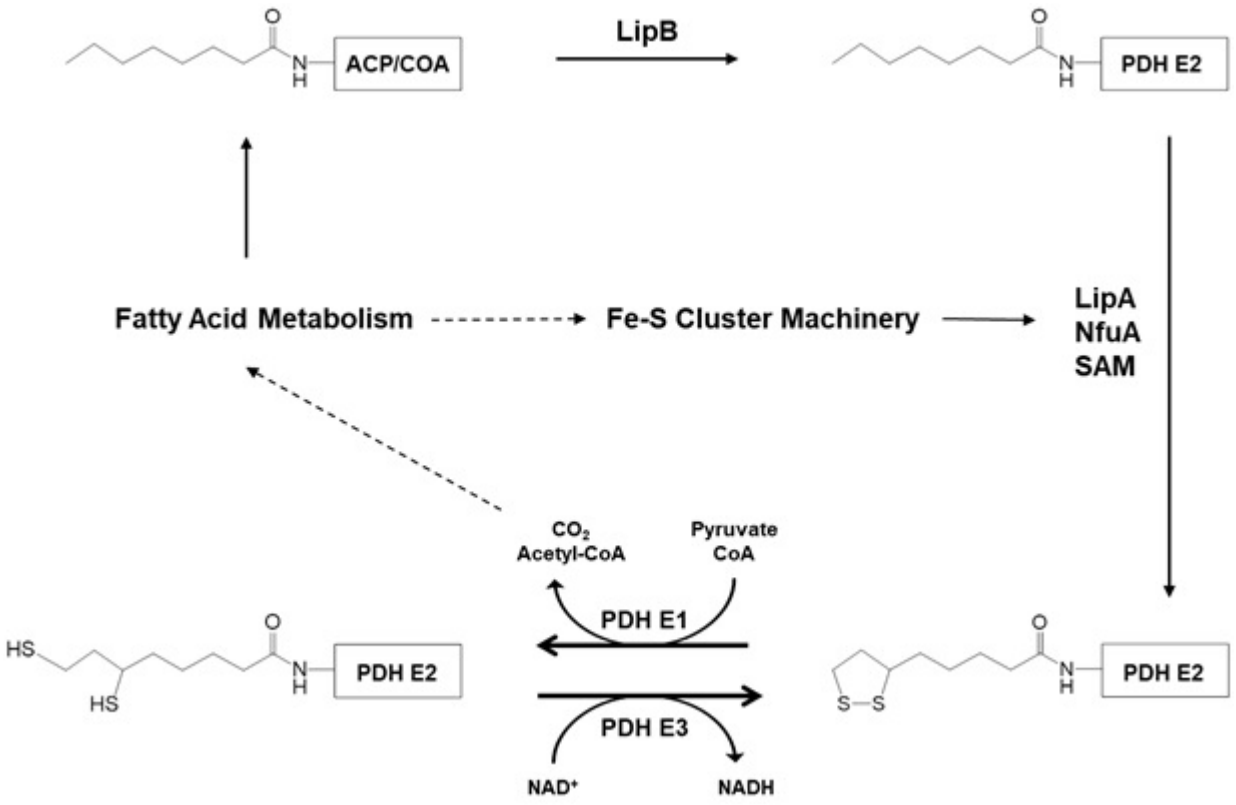

Figure 1.1: The canonical mechanism for lipoate biosynthesis and lipoylation of the pyruvate dehydrogenase (PDH).
This mechanism is also the likely mechanism by which the *Plasmodium* apicoplast lipoylation machinery lipoylates the apicoplast PDH.

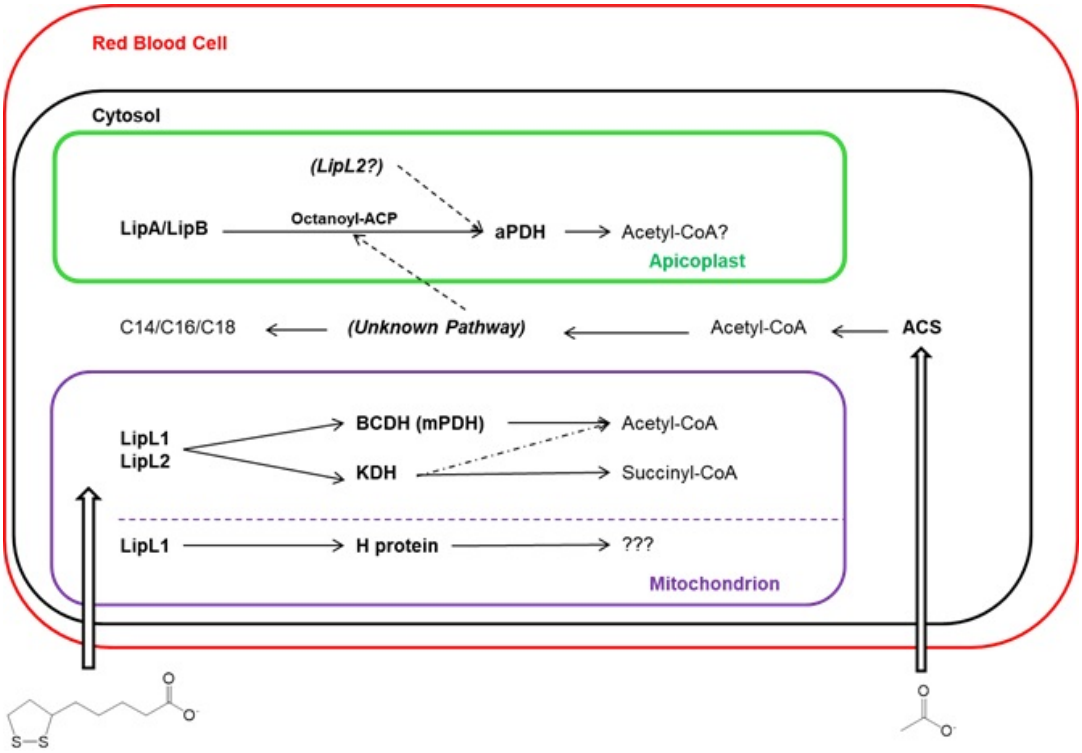

Figure 1.2: Lipoylation in the blood stage of *Plasmodium*. In the apicoplast of the parasite, LipB transfers the octanoyl group (generated by an unknown source, but possibly derived from acetate metabolism) from ACP to the E2 subunit of the apicoplast PDH, followed by sulfuration by LipA. The mitochondrion of the parasite scavenges exogenous lipoate, and lipoylates the H protein under relatively oxidative conditions, and works in concert with LipL2 to lipoylate the BCDH and KDH E2 subunits under normal conditions.

Chapter 2

Mitochondrial Lipoylation is essential in *P. falciparum*

2.1 Abstract

Lipoate is a redox active cofactor that is covalently bound to key enzymes of oxidative metabolism. *Plasmodium falciparum* is auxotrophic for lipoate during the intraerythrocytic stages, but it is not known whether lipoate attachment to protein is required, nor whether lipoate is required in a specific subcellular compartment of the parasite. To address these questions, we used an enzyme called lipoamidase (Lpa) as a probe of lipoate metabolism. Lpa was first described in *Enterococcus faecalis*, and specifically cleaves protein-bound lipoate, inactivating enzymes requiring this cofactor. Enzymatically active Lpa could be expressed in the cytosol of *P. falciparum* without any effect on protein lipoylation or parasite growth. Similarly, Lpa could be expressed in the apicoplast, and although protein lipoylation was reduced, parasite growth was not inhibited. By contrast, while an inactive mutant of Lpa could be expressed in the mitochondrion, the active enzyme could not. We designed

an attenuated mutant of Lpa and found that this enzyme could be expressed in the parasite mitochondrion, but only in conjunction with a chemical bypass system. Our study shows that lipoate attachment to proteins in the mitochondrion is critical for the survival of malaria parasites.

2.2 Introduction

Lipoic acid (6,8-thiooctanoic acid) is a metabolic cofactor required for the decarboxylation of alpha-ketoacids and the amino acid glycine in oxidative and one carbon metabolism (Cronan et al., 2005). *Plasmodium spp*. have four metabolic complexes that use the lipoic acid cofactor: pyruvate dehydrogenase (PDH), which resides in the apicoplast, and the alpha-ketoglutarate dehydrogenase (KDH), branched-chain alpha-ketoacid dehydrogenase (BCDH), and the glycine cleavage complex (GCV), all of which reside in the mitochondrion (Afanador et al., 2014; Allary et al., 2007). These complexes are generally composed of three enzymes named E1, E2, and E3, with the lipoic acid cofactor covalently bound to one or more conserved lysine residues on the E2 subunit. The GCV differs from other lipoylated complexes in that its substrate is not an alpha ketoacid, but instead is the amino acid glycine, and its subunits are canonically called the T, H, P, and L proteins, of which the H protein is lipoylated and non-catalytic (Douce et al., 2001). However, there appears to be no evidence of the L or P proteins in malaria parasites.

The presence of lipoylated proteins in the apicoplast and mitochondrion indicates that both organelles possess functional lipoylation pathways. There are four enzymes encoded in the *P. falciparum* genome that are involved in lipoic

acid acquisition: lipoate synthase (*Pf*LipA, PF3D7_1344600), lipoate transferase (*Pf*LipB, PF3D7_0823600), and two enzymes with homology to lipoate ligases, designated LipL1 (PF3D7_1314600) and LipL2 (PF3D7_0923600). *Pf*LipA and *Pf*LipB reside in the apicoplast (Wrenger and Müller, 2004) and functionally complement their *E. coli* homologs, showing that *P. falciparum* possesses a lipoate synthesis pathway (Allary et al., 2007). LipL1 and LipL2 work in concert to lipoylate the H protein and the E2 subunits of the BCDH and KDH. The two ligases also appear to have different subcellular localizations: LipL1 localizes exclusively to the mitochondrion (Wrenger and Müller, 2004); by contrast, LipL2 appears to partition between the apicoplast and the mitochondrion, but its distribution between these organelles is not uniform in the parasite population (Günther et al., 2007). Uptake and incorporation of ^{35}S-lipoic acid by blood stage parasites shows that despite the potential presence of LipL2 in the apicoplast, only mitochondrial proteins are lipoylated by scavenging (Allary et al., 2007). Collectively, these results show that there are organelle-specific mechanisms of lipoate acquisition in *P. falciparum*, in which lipoate scavenging occurs exclusively in the mitochondrion, and lipoylation in the apicoplast relies on lipoate synthesis.

Although lipoylation in the apicoplast may be dispensable in the blood-stage of the parasite (Cobbold et al., 2013; Laine et al., 2015), growing evidence indicates that lipoate scavenging for use in the mitochondrion is likely vital to the *P. falciparum* erythrocytic stage parasites. Treatment of parasite cultures with lipoate analogs 8-bromooctanoate or 6,8-dichlorooctanoate result in decreased mitochondrial lipoylation and inhibited parasite growth (Afanador et

al., 2014; Allary et al., 2007). This appears likely due to ligation of the analog to the mitochondrial substrates, which can be mitigated by excess lipoate, indicating that lipoate scavenging and attachment is important for survival. Consistent with these findings is the report that LipL1 is refractory to deletion in the murine malaria parasite, *P. berghei* (Günther et al., 2007; Wang et al., 2017).

In this study, we exploited the partitioned nature of lipoate metabolism in *P. falciparum* to probe the effects of inactivating lipoylated complexes in the apicoplast and mitochondrion with an enzyme called lipoamidase. Lipoamidase (Lpa) is an enzyme first described in *Enterococcus faecalis* that can cleave lipoate from lipoylated proteins (Jiang and Cronan, 2005). Our work in *E. coli* demonstrated that this can be performed in vivo to induce inactivation of alpha-keto acid complexes, resulting in a growth defect (Spalding and Prigge, 2009); related observations have been made with a new class of lipoamidases related to the sirtuin family of proteins that appears to be conserved between human cells and bacteria (Mathias et al., 2016; Rowland et al., 2017). The mycobacteriophage integrase system (Spalding et al., 2010) was used to generate *P. falciparum* cell lines containing integrated Lpa transgene constructs targeted to the cytosol, apicoplast, and mitochondrion. Expression of active Lpa in the cytosol did not affect parasite growth or lipoylation, demonstrating that no significant lipoate metabolism occurs in the cytosol. Parasites expressing active Lpa in the apicoplast showed a significant, but not total, decrease in lipoylated PDH E2 relative to cells expressing catalytically inactive Lpa, and appeared unaffected by this change. In contrast, we were unable to

produce parasite lines expressing fully active Lpa in the mitochondrion. We were able to express a catalytically dead Lpa in this organelle, but a catalytically attenuated Lpa mutant could only be expressed in the mitochondrion in conjunction with chemical supplementation of 5 mM acetate in the parasite growth medium. These results demonstrate that mitochondrial lipoylation is essential during the blood stages of parasite development, and provide insight into the essential roles that lipoylated proteins play in the mitochondrion.

2.3 Results

2.3.1 *P. falciparum* can express the bacterial gene encoding Lpa.

Lpa has been used *in vivo* as an inducible probe of alpha-ketoacid dehydrogenase inactivation in *E. coli* (Spalding and Prigge, 2009), and the suitability of Lpa as an *in vivo* probe hinges on the ability of the organism under study to express the bacterial enzyme in an active form. To evaluate whether *P. falciparum* can express Lpa, we used mycobacteriophage integrase mediated recombination to generate parasite lines containing an integrated copy of the gene (Spalding et al., 2010) encoding either active Lpa or Lpa inactivated by mutations of active site residues K159A and S259A (KSA). Lpa constructs were tagged at the C-terminus with a hemagglutinin (HA) epitope tag. This generated the cell lines Lpa and KSA. Plasmid integration at the attB locus was confirmed by PCR (**Fig. 2.1A, B**).

Expression of Lpa was determined by SDS-PAGE fractionation of whole cell lysates, followed by western blot with monoclonal antibodies specific for

HA. In both cell lines, a single HA-tagged protein of approximately 100 kDa is present (**Fig. 2.1C**) at roughly equivalent levels. Untagged Lpa has previously been reported to migrate at 97 kDa, despite a predicted molecular weight of 79 kDa (Jiang and Cronan, 2005); therefore, the bands identified by anti-HA western blot are consistent with the empirical size of Lpa. In the absence of N-terminal leader sequences, Lpa is expected to localize to the cytosol. To confirm the localization of Lpa, fixed Lpa and KSA parasites were probed with antibodies specific for HA and co-stained with anti-aldolase antibodies to delineate the cytosol and DAPI to identify the nucleus. The signal from HA occupies most of the parasite cell and is coincident with aldolase staining, but exclusive of the nucleus (**Fig. 2.2A**). Taken together, these results show that Lpa and the catalytically dead KSA mutant can be expressed in cytosol of *P. falciparum* parasites.

2.3.2 Lpa expressed in the cytosol is active and does not inhibit parasite growth

Lipoylated proteins and the enzymes involved in lipoylation have been localized exclusively to the apicoplast and mitochondrion, so expression of active lipoamidase in the cytosol was not expected to perturb protein lipoylation. To test this hypothesis, we compared protein lipoylation in Lpa and KSA parasites by assaying apicoplast lipoylation using western blotting with an antibody recognizing lipoylated proteins and assaying mitochondrial lipoylation by using autoradiography with cultures supplemented with ^{35}S-radiolabeled lipoate. When cell pellets were lysed under denaturing conditions we found that both parasite lines showed similar levels of lipoylation relative to the

HSP70 loading control (**Fig. 2.2B**, left panels), indicating that lipoylation was not affected by Lpa expression. We then assayed Lpa activity by lysing identical samples under non-denaturing conditions, followed by incubation at 30°C for 15 minutes. Among the Lpa lysates, no detectable lipoylation remained after incubation. By contrast, protein lipoylation was maintained in lysates of the catalytically dead KSA mutant (**Fig. 2.2B**, right panels). This demonstrates that Lpa is expressed by *P. falciparum* in an active form and that Lpa can recognize and cleave lipoate from all four P. falciparum lipoylated proteins if given access.

We then compared the growth of Lpa and KSA parasite lines to the parental line to determine whether expression of the active enzyme resulted in a growth phenotype. Expression of either Lpa or KSA did not result in discernible differences in growth when compared to the parental line (**Fig. 2.2C**). Coupling this observation to the unchanged lipoylation state of the Lpa expressing line suggests that there is no lipoylated protein in the cytosol that would be important for parasite growth, nor is there off-target, deleterious activity by Lpa in the cysotol.

2.3.3 Reduced lipoylation of the apicoplast pyruvate dehydrogenase does not impair parasite growth

In order to determine whether lipoylated PDH is essential for the growth of blood stage *P. falciparum*, we sought to expresss Lpa in the apicoplast. To direct Lpa to the apicoplast, the first 55 amino acids of the acyl carrier protein (ACP_55), which was previously shown to direct GFP to the apicoplast (Waller et al., 2000), was appended to the Lpa and KSA constructs. Initial attempts to

express ACP_{55}Lpa and ACP_{55}KSA using the strong calmodulin promoter were unsuccessful, perhaps because overexpression of apicoplast proteins by this promoter is poorly tolerated - a phenomenon that we have observed before (Gisselberg et al., 2013). Switching to the lower strength RL2 (ribosomal L2 protein) promoter (Nina et al., 2011) allowed us to generate parasite lines for both constructs. Integration was confirmed by PCR (**Fig. 2.3A**), and anti-HA western blot analysis of whole cell lysates from both parasite lines recognized a doublet characteristic of apicoplast-localized proteins (van Dooren et al., 2002) in which the lower band corresponds to the expected 100 kDa size of mature Lpa after import into the apicoplast (**Fig. 2.3B**). As in the cytosolic constructs, western blotting suggests relatively equivalent expression of the two constructs. To determine the subcellular localization of the lipoamidase proteins, we performed immunofluorescence assay (IFA) on fixed ACP_{55}Lpa and ACP_{55}KSA parasites. The signal from anti-HA co-localizes with the apicoplast marker anti-ACP (**Fig. 2.4A**), demonstrating that both ACP_{55}Lpa and ACP_{55}KSA localize primarily to the apicoplast.

The effect of ACP_{55}Lpa expression on lipoylation was determined by western blot and autoradiography. Western blotting revealed that PDH lipoylation was reduced in ACP_{55}Lpa compared to ACP_{55}KSA parasites, although some lipoylation persists. Autoradiography of parasites cultured in the presence of ^{35}S-lipoate shows that mitochondrial lipoylation is largely unaffected (**Fig. 2.4B**, right panels). We were curious whether low activity of ACP_{55}Lpa (due to improper protein folding, modification of the N-terminus, or some other

factors) was responsible for the persistence of some lipoylated PDH. To investigate this, we analyzed the activity of ACP_{55}Lpa in parasite extracts and found that ACP_{55}Lpa rapidly reduced lipoylation of all four parasite proteins to below the limit of detection (**Fig. 2.4**, left panels). This result shows that ACP_{55}Lpa is capable of completely delipoylating PDH, but in the apicoplast, some factors prevent it from doing so efficiently, such as the environmental conditions or robust lipoylation activity by the lipoate synthesis machinery. Ultimately, reduction of lipoylation in the apicoplast appears to have little effect on the parasite, since expression of Lpa does not impart a growth phenotype when compared to KSA expression or the parental line (**Fig 2.4C**).

2.3.4 Lipoamidase is toxic when expressed in the mitochondrion

In order to target lipoamidase to the mitochondrion, the cytosolic constructs were again modified, this time to contain an N-terminal mitochondrial targeting motif using the first 33 amino acids from LipL1 ($L1_{33}$). In repeated transfections, we were able to generate parasite lines expressing mitochondrially targeted $L1_{33}$KSA, however, multiple attempts at expressing $L1_{33}$Lpa failed (**Table 2.1**). These results are consistent with the hypothesis that one or more of the lipoylated proteins found in the mitochondrion are essential for parasite growth. We attempted to demonstrate this phenomenon by using a conditional destabilization approach and a chemical bypass approach. In the first approach we used the destabilization domain (DD) developed by Armstrong and Goldberg (Armstrong and Goldberg, 2007) to generate parasite lines expressing a destabilized lipoamidase. As shown in **Table 2.1**,

we were not able to generate parasites expressing lipoamidase with either an N-terminal or a C-terminal DD. Acetate and succinate supplementation has been described as a means of bypassing the essential lipoylated enzymes (KDH and PDH) in *E. coli* (Jiang and Cronan, 2005). Even with chemical supplementation (5 mM acetate) we were still not able to generate parasites expressing $L1_{33}$Lpa (**Table 2.1**).

2.3.5 Lipoylated proteins in the mitochondrion are essential for parasite survival

Cell-free activity assays, such as that shown in **Figure 2.2B** demonstrate that lipoamidase can cleave lipoate from all parasite proteins in a surprisingly short period of time. We hypothesized that Lpa activity may be too high to effectively control with the conditional tools available for *P. falciparum* genetics, all of which have limited dynamic ranges. We considered using low strength promoters to drive Lpa expression, however, this approach makes it difficult to identify the expressed protein and confirm its localization. Instead, we attempted to attenuate the activity of lipoamidase with a series of active site mutations. We made a homology model of the *E. faecalis* lipoamidase based on the structure of a hydrolase (PDB code 3A2Q) (Yasuhira et al., 2010) with 27% sequence identity and designed active site mutations to attenuate Lpa activity. Point mutations were designed to interfere with hydrogen bonds orienting the catalytic lysine K159 (S236A, S236C and S236G), to disrupt stabilization of the amide carbonyl (A256G, Y375F), and to interfere with the hypothetical position of the lipoate dithiolan ring (W210F) (**Fig. 2.8**). Quantification of lipoamidase activity using an *E. coli* bioassay identified S236A as the most

attenuated mutant with a 100-1000 fold decrease in catalytic activity (**Table 2.3, Fig. 2.9**). This variant was then cloned into the same transfection vector as $L1_{33}$Lpa and named $L1_{33}$S236A.

The S236A lipoamidase variant was successfully expressed under supplementation with 5mM acetate. As with the other constructs, PCR for attB integration and western blotting was used to confirm expression (**Fig. 2.5A** and **2.5B**). Unlike the gene products targeted to the cytosol and the apicoplast, there was a marked difference in protein levels in the mitochondrion as observed by western blot. The parasite appears to have significantly decreased levels of the $L1_{33}$S236A construct when compared to $L1_{33}$KSA, even though they are driven by the same regulatory elements. In addition, there appear to be some degradation of both gene products, unlike what was demonstrated for lipoamidase expressed in the cytosol or the apicoplast. Immunofluorescence was used to determine mitochondrial localization using MitoTracker Red. PCC values and PDM images show that lipoamidase is successfully expressed in the mitochondrion, but there remains some amount of protein that is outside the mitochondrion (**Fig. 2.6A**). This holds true for $L1_{33}$KSA expression as well, suggesting that this finding is likely an artifact of the overexpression system rather than a specific effect due to lipoamidase activity.

Western blotting reveals that the apicoplast PDH remains lipoylated in both the $L1_{33}$S236A and $L1_{33}$KSA parasite lines, consistent with these constructs having no impact on apicoplast protein lipoylation. By contrast, there is a significant reduction in lipoylation of all three lipoate-dependent proteins found in the mitochondrion when compared to either the loading control or

the apicoplast PDH lipoylation (**Fig. 2.6B**). These results show that the attenuated S236A lipoamidase mutant is sufficiently active to reduce, but not abolish the lipoylation of mitochondrial proteins. Parasite growth curves show that the $L1_{33}$KSA line demonstrates a minor growth defect when compared to the parental line - a difference that may be due to the overexpression of $L1_{33}$KSA by the strong calmodulin promoter. Importantly, acetate supplementation does not affect the growth of either the parental or the $L1_{33}$KSA parasite lines (**Fig. 2.6C**). The growth of the $L1_{33}$S236A parasite line, however, is dependent on acetate supplementation and this line cannot be maintained in the absence of acetate. Taken together, these results demonstrate that lipoamidase cleaves lipoate from all three mitochondrial proteins resulting in parasites that cannot grow without acetate supplementation.

2.4 Experimental Procedures

2.4.1 Generation of the lipoamidase constructs in the pLZ *E. coli* expression vector

Primers used for this work are listed in **Table 2.2**. We generated a series of lipoamidase constructs in the pLZ expression vector (Allary et al., 2007) (MalE gene of pMAL_cHT (Muench et al., 2003) replaced with the amino acids MRGS). The pLZ vector allows us to evaluate the activity of any lipoamidase construct in *E. coli* as previously described (Spalding and Prigge, 2009). We began with pLZ plasmid pMS007, which contains the lipoamidase gene from *Enterococcus faecalis* strain V583 (Spalding and Prigge, 2009) and inserted a hemagglutinin (HA) tag by site-directed mutagenesis using the primers

HA.F and HA.R, generating plasmid pMS011 encoding the construct Lpa. To generate Lpa containing a mitochondrial targeting sequence, the nucleotide sequence encoding the first 33 amino acids of the *P. falciparum* mitochondrial protein lipoate ligase 1 (LipL1, PF13_0083) was amplified from *P. falciparum* cDNA using the primers $L1_{33}$BamHI.F and $L1_{33}$BamHI.R. This amplicon was inserted into the *Bam*HI site at the 5' end of the Lpa gene in plasmid pMS011 to create plasmid pMS030, which encodes the construct $L1_{33}$Lpa. Apicoplast-targeted lipoamidase, was produced by a similar method, except that the first 55 amino acids of the acyl carrier protein (ACP, PFB0385w) which encode the signal peptide and apicoplast transit peptide, were amplified from the plasmid pSP002 (Waters et al., 2002) using the primers ACP_{55}BamHI.F and ACP_{55}BamHI.R. Ligation of the digested amplicon into pMS011 produced the plasmid pMS021, containing the construct ACP_{55}Lpa.

Active site double mutants were engineered as controls for the active Lpa, although a single mutation of any of the catalytic triad residues to alanine was sufficient to inactivate the enzyme for in vivo studies in *E. coli* (Spalding and Prigge, 2009). The plasmids pMS011, pMS021, and pMS030 were mutagenized to contain the K159A/S259A double mutant by consecutive rounds of site-directed mutagenesis with primer sets K159A.F/K159A.R and S259A.F/S259A.R. The resulting plasmids: pMS015, pMS023, and pMS033, contain the double mutant constructs denoted KSA, ACP_{55}KSA, and $L1_{33}$KSA, respectively. We generated a series of lipoamidase mutants based on the $L1_{33}$Lpa construct with the goal of attenuating lipoamidase enzymatic activity. Site directed mutagenesis was performed on pMS030 using primer pairs

S236A.F/S236A.R, S236C.F/S236C/R, S236G.F/S236G.R, W210.F/W210.R, A256G.F/A256G.R, and Y375.F/Y375.R to produce plasmids pLZ-LpaS236A, pLZ-LpaS236C, pLZ-LpaS236G, pLZ-LpaW210F, pLZ-LpaA256G and pLZ-LpaY375F.

2.4.2 Evaluation of lipoamidase mutants with attenuated activity

The pLZ constructs described above, as well as pMS030 and pMS033, were transformed into lipoylation deficient JEG3 cells (Gisselberg et al., 2013) in order to reduce background lipoylation signal in cell lysate experiments. Cells were induced with 0.4 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) overnight at 20°C, and lysed in BugBuster (EMD Millipore), 1 mg/mL lysozyme, 2.5 μg/mL DNAse I for 10 minutes, and used immediately or frozen at -80°C. Lysates were analyzed by western blot for the presence of HA tagged proteins to determine relative expression levels for each construct using ImageJ quantification. The lysates were then diluted in reaction buffer described below to normalize protein levels for comparative enzymatic assays.

The H protein from *Bacillus subtilis* was used as substrate for the lipoamidase experiments. The coding region was amplified from genomic DNA using primers BsHprot.BamHI.F and BsHprot.SalI.R and cloned into the pMAL-cHT expression plasmid using *Bam*HI and *Sal*I. BL21-Star (DE3) cells (Invitrogen) were transformed with this plasmid and the pRIL plasmid isolated from BL21-CodonPlus-RIL cells (Agilent). Cells were induced at midlog phase with 0.4 mM IPTG and harvested after growth at 20° C after 10 hours. The H protein was purified by affinity chromatography and ion exchange using MBPTrap

and HiTrap Q columns (GE Healthcare).

Lipoamidase activity assays were carried out in 200 mM HEPES and 100 mM NaCl at pH 7.5 with 50 nM purified lipoylated H protein from *Bacillus subtilis* serving as the substrate for the reaction. Diluted lysates were added to each reaction and the reaction mixture was incubated at room temperature for 30 minutes. Reactions were quenched with addition of an equivalent volume of 6X Laemmli buffer and boiled for four minutes and vortexed for one minute performed twice for a total of ten minutes. Lipoylated H protein was detected by western blot probed with 1:5,000 rabbit anti-lipoate antibodies (Afanador et al., 2017) and 1:5,000 donkey anti-rabbit IgG horseradish peroxidase (HRP) secondary antibody (GE Healthcare). Quantiation of lipoylation signal was performed by ImageJ analysis.

2.4.3 Construction of *Plasmodium falciparum* expression plasmids

For expression in *P. falciparum*, lipoamidase constructs were cloned into the vector pLN-ENR-GFP (Nkrumah et al., 2006) obtained from the MR4, or a derivative of this vector, pRL2 in which the strong calmodulin promoter was replaced with the weaker RL2 promoter (Nina et al., 2011). Lpa constructs were PCR amplified from the abovementioned plasmid templates using the reverse primer HAAflII.R, and the following forward primers: LpaAvrII.F for plasmids pMS011 and pMS015, ACP_{55}AvrII.F for plasmid pMS021, and $L1_{33}$AvrII.F for plasmids pMS030 and pMS033. The resulting amplicons were digested with *Avr*II and *Afl*II followed by ligation into the same endonuclease sites in pLN-ENR-GFP. This produced six plasmids: pMS039 (encoding

Lpa), pMS040 (encoding KSA), pMS026 (encoding ACP_{55}Lpa), pMS036 (encoding $L1_{33}$Lpa), pMS037 (encoding $L1_{33}$KSA) and pLN-$L1_{33}$S236A (encoding $L1_{33}$S236A). Amplicons from plasmids pMS021 and pMS023 were also inserted into digested pRL2 to produce plasmids pMS049 and pMS050 which encode ACP_{55}Lpa and ACP_{55}KSA driven by the RL2 promoter.

2.4.4 Parasite culture, transfection and harvesting

P. falciparum asexual blood stage cultures were maintained at 2% hematocrit in complete medium consisting of RPMI 1640 medium with L-glutamine (USBiological Life Sciences) supplemented with 10% human serum or 5 g/L AlbuMax II (Thermo Fisher), 20 mM HEPES, 23 mM $NaHCO_3$, and 0.09 mM hypoxanthine. Transfections were performed as described previously (Spalding et al., 2010); briefly, 400 μL of blood was washed with 5 mL of CytoMix and resuspended in 400 μL of CytoMix and 100 μg of the pINT plasmid encoding mycobacteriophage Bxb1 integrase (Nkrumah et al., 2006) and 100 μg of the appropriate parasite expression vector. Drug selection was initiated 1-2 days after transfection. Where described, parasites lines were also supplemented with 5 mM sodium acetate.

For western blot, autoradiography, and genomic DNA extraction, red blood cells from P. falciparum cultures were pelleted by centrifugation at 500 g for five minutes before lysis of RBCs with 0.2% saponin for three minutes. The reaction was quenched with ice cold PBS, and the parasites were pelleted by centrifugation at 3,000 g for ten minutes followed by three washes with ice cold PBS. The parasite pellets were used immediately or stored at -80° C.

2.4.5 Immunofluorescence microscopy

For mitochondrial staining, parasite culture was treated with 75nM MitoTracker Red CMX-Ros (Invitrogen) for 30 minutes in fresh media prior to fixation. All cultures were fixed in 4% paraformaldehyde/0.0075% glutaraldehyde onto poly-lysine treated slides for thirty minutes. Cells were permeabilized for 10 minutes in 1% Triton X-100, remaining aldehydes were reduced with 0.1 mg/mL $NaBH_4$ for 10 minutes and cells were blocked for 2 hours in 3% BSA. Parasite cytosol was stained with 1:2000 mouse anti-aldolase, the apicoplast was stained with 1:1,000 rabbit anti-ACP (Gallagher and Prigge, 2009) and lipoamidase was stained with 1:500 rat anti-HA mAb 3F10 (Roche) overnight. After treatment with primary antibodies, slides were washed, treated with cognate secondary antibodies [1:2,000 rabbit anti-Mouse IgG Alexa Fluor 594, 1:2,000 goat anti-Rabbit IgG Alexa Fluor 594 or 1:2,000 goat anti-Rat IgG Alexa Fluor 488 (Invitrogen)], washed and mounted with Prolong Gold antifade reagent with DAPI (Invitrogen). Microscopy images were taken with the Zeiss Axio Imager.

2.4.6 *In vivo* radiolabeling of mitochondrial substrates

^{35}S-labeled lipoate was prepared as previously described (Allary et al., 2007). Labeling with 0.2 μCi of ^{35}S-radiolabeled lipoate was initiated in 1% hematocrit cultures with an initial parasitemia of 2%. Cultures were fed supplemented media and blood films were made to monitor parasites every day for 48 hours.

2.4.7 Western blotting and autoradiography

Parasite pellets as described above were resuspended in RIPA buffer to determine lipoamidase activity in cell lysates, and the reaction was quenched by addition of Laemmli loading buffer. Otherwise, pellets were resuspended in NuPAGE LDS sample buffer and boiled and vortexed for 10 minutes. Proteins were resolved by SDS-PAGE on 4-12% gradient gels and transferred to nitrocellulose membranes. For autoradiography, membranes were dried overnight and autoradiography was used to detect 35S lipoate incorporation. For Western blotting, membranes were blocked and probed with rat anti-HA primary antibodies (1:5000) to detect lipoamidase, rabbit anti-lipoate (1:5,000) to detect lipoylation of the apicoplast PDH E2 subunit, and either mouse anti-HSP70 or mouse anti-aldolase (1:10,000) for loading controls.

2.5 Discussion

2.5.1 Mitochondrial lipoylation generates an essential acetyl-CoA pool

Lipoic acid is a metabolic cofactor that is acquired in an unusual manner by the parasite: although it is synthesized in the apicoplast, mitochondrial proteins are modified with host-derived lipoate. Our experiments demonstrate a dose-dependency between catalytic ability of lipoamidase in the parasite mitochondrion and parasite survival: as catalytic ability increases (from the inactive KSA variant, the attenuated S236A variant, and the wild-type enzyme), parasite survival decreases, including complete intolerance of

wild-type lipoamidase expression in the mitochondrion, even with acetate supplementation. These observations indicate that *P. falciparum* is auxotrophic for lipoate in the blood stage of the parasite life cycle. This means that the some combination of the mitochondrial lipoate ligases and the lipoylated substrates are essential for the parasite.

The growth inhibition imparted by expression of mitochondrial S236A and its partial rescue clarifies one, crucial, mitochondrial biochemical pathway. The current consensus regarding the gene product previously identified as a mitochondrial branched chain alpha-ketoacid dehydrogenase (BCDH) is that it is more likely a mitochondrial pyruvate dehydrogenase (PDH), and our experiments support this claim. Bioinformatic analysis reveals an absence of enzymes upstream of the putative BCDH, while metabolomic work confirms the parasite's inability to catabolize branched chain amino acids (Cobbold et al., 2013; Oppenheim et al., 2014), calling into question the existence of a true BCDH. The parasite does have a functional pyruvate dehydrogenase the bridges glycolysis to the citric acid cycle (MacRae et al., 2013). Knockout of the apicoplast resident PDH resulted in no severe growth phenotype and no change in the parasite's pool of acetyl-CoA (Cobbold et al., 2013; Günther et al., 2007), making the case that the apicoplast PDH is not the enzyme chiefly responsible for the conversion of glucose-derived pyruvate to acetyl-CoA. Knockout of the E1 subunit of the BCDH in *P. berghei*, however, results in a severe growth phenotype that is also partially rescued by *ex vivo* acetate supplementation, where this rescue is likely mediated by the putative acetyl-CoA synthetase that can convert acetate to acetyl-CoA (Cobbold et al., 2016). Our

experiments mirror these observations: by drastically reducing mitochondrial lipoylation with S236A, it appears that mitochondrially derived acetyl-CoA generation is concomitantly reduced, with acetate supplementation providing a partial metabolic bypass through the cytosolic acetyl-CoA synthetase (**Fig. 2.7**). These observations show that the parasite's chief source of acetyl-CoA is from the mitochondrion, that the BCDH is likely a mitochondrial PDH that is responsible for generating acetyl-CoA from pyruvate, and that this source of acetyl-CoA is necessary for parasite growth.

It is unclear if reducing lipoylation of the KDH contributes to the growth defect imparted by mitochondrial expression of S236A. KDH biochemistry may be dispensable and the parasite is able to biochemically compensate for the absence of this pathway (Ke et al., 2015), suggesting that preventing the conversion of alpha-ketoglutarate to succinyl-CoA causes no growth phenotype to the parasite. However, the KDH E1 subunit is, at least *in vitro*, competent in performing PDH biochemistry, decarboxylating both alpha-ketoglutarate and pyruvate at reasonably comparable rates (Chan et al., 2013). Therefore, both the BCDH and the KDH may be contributing to the acetyl-CoA pool under different circumstances. Either way, our experiments demonstrate that it is reduction of acetyl-CoA generation that is inhibiting parasite growth.

2.5.2 The H protein of the parasite may be essential

It remains unclear why expression of the inactive mutant is not completely tolerated in the parasite. The fact that acetate supplementation does not appear to rescue against this growth phenotype suggests that this inhibition is

independent of acetyl-CoA generation. It is possible that this is merely an artifact of overexpression of an exogenous gene in the parasite mitochondrion interfering with normal trafficking or proteostasis in the parasite mitochondrion. This phenomenon appeared to be the case for the apicoplast as well since expression of $ACP_{55}KSA$ was only successful after switching to the lower strength RL2 promoter. Acetate rescue is insufficient to fully rescue growth inhibition in parasites expressing mitochondrial S236A, even to the levels of acetate-supplemented KSA parasite line. This may be explained by the inability of the cytosolic bypass to sufficiently produce acetyl-CoA.

Another possibility for both the S236A partial rescue phenotype is inhibition of the H protein. Bioinformatic analysis reveals that the parasite codes only two of the four proteins required for the glycine cleavage system: the T and H proteins. Biochemical work (Varadarajan et al., 2014) demonstrated that the parasite does not appear to perform canonical glycine cleavage, and both their attempts and our attempts to knock out the gene in *P. berghei* and *P. falciparum* were unsuccessful. These findings suggest that there is a non-canonical, essential role of the H protein in blood stage malaria parasites. Whatever its function, it is unlikely that the H protein has any contribution to the synthesis of acetyl-CoA in the mitochondrion, and thus it is likely that acetate supplementation will not bypass loss of H protein activity. Presumably, H protein lipoylation would be crucial for its function, potentially explaining why acetate supplementation does not fully bypass the activity of the S236A lipoamidase mutant.

2.5.3 Complete lipoylation in the apicoplast is dispensible

In many replicate experiments, such as the one shown in **Figure 2.4B**, right panels, lipoamidase was not able to completely cleave lipoate from PDH in the apicoplast. Initially, we thought that this could be a consequence of using the lower strength RL2 promoter to drive the apicoplast constructs, or that one of the two lipoylation domains found in PDH might be resistant to lipoamidase activity. Alternatively, factors associated with apicoplast trafficking such as the presence of the N-terminal apicoplast targeting peptide or refolding of the trafficked protein could affect activity. All of these possibilities are discounted by the experiment shown in **Figure 2.4B**, left panels, which demonstrates that the ACP_{55}Lpa construct is able to rapidly cleave lipoate from PDH and the other lipoylated proteins when the organellar contents are mixed by cell lysis. This indicates that there is something specific about the apicoplast that limits lipoamidase activity. One possibility is that the conditions in the apicoplast, such as the pH and redox potential (Mohring et al., 2017) or the presence of inhibitory compounds, could limit lipoamidase activity. If inhibitory compounds or conditions exist, their effects are reversed by cell lysis. Another possibility is that the LipB/LipA apicoplast lipoylation machinery simply works faster than lipoamidase activity. This would involve rapid action of the octanoyl transferase LipB followed by addition of the lipoate sulfurs by the lipoate synthase LipA. It is hard to imagine rapid catalysis by LipA since the protein donates sulfur atoms from its iron-sulfur cofactor to lipoate and must be repaired prior to each round of catalysis. Although it seems unlikely, apicoplast lipoylation remains a possibility in part due to

uncertainty about how this pathway functions. Deletion of the LipB protein, which should be essential for apicoplast lipoylation, results in parasites that still have some residual PDH lipoylation in both *P. falciparum* and *P. berghei* (Falkard et al., 2013; Günther et al., 2007).

Our experiments demonstrate that reducing apicoplast lipoylation results in no discernible growth phenotype. This observation falls in line with the above mentioned knockout experiments of the apicoplast lipoylation machinery and knockout of several subunits of the apicoplast PDH in several *Plasmodium* species (Cobbold et al., 2013; G"unther et al., 2007; Laine et al., 2015; Pei et al., 2010a). Although PDH activity is essential for liver stage malaria parasites (Pei et al., 2010b), its activity in blood stage parasites is puzzling. The apicoplast resident PDH should be fully competent to perform catalysis based on in vitro experiments (Chan et al., 2013), but it appears that it may not be performing this reaction at a detectable rate *in vivo* (Cobbold et al., 2013). Certainly the substrate pyruvate is available in the apicoplast since this is also a substrate for the essential isoprenoid biosynthesis pathway. If PDH activity is not needed for blood stage parasites, then why is it produced and maintained in a lipoylated state?

2.5.4 Lipoamidase as a tunable tool to probe lipoylation

Lipoamidase has proven to be an effective tool to probe in vivo lipoylation. The enzyme appears to lack strong substrate specificity, recognizing lipoylated proteins from *E. coli, B. subtilis* and *P. falciparum*, opening the door to the possibility of using this tool in other organisms. The two mutants generated

in this project serve as a control for protein expression (WT Lpa vs. KSA) and a means of probing cells with different levels of lipoamidase activity (WT Lpa vs. attenuated mutants such as S236A). Adding this to inducible and knockdown systems available in other organisms offers a wide array of options to knock down in vivo lipoylation, especially in systems in which the full array of lipoylation machinery has not yet been discovered. Our experiments in *P. falciparum* demonstrate that mitochondrial lipoylation is essential and implicate the BCDH and H protein as being responsible for this phenomenon. These results explain why lipoate scavenging is essential for parasite survival and imply that the lipoate attachment enzymes LipL1 and LipL2 will also be required for blood stage parasite survival. Overall, these findings provide new insight into key aspects of mitochondrial metabolism in malaria parasites.

2.6 Acknowledgments

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2.8 Tables

Localization	Cell Line	Transfections attempted	Lines with successful expression
Cytosol	Lpa	2	2
	KSA	2	2
Apicoplast	ACP_{55}Lpa	2	2
	ACP_{55}KSA	2	2
Mitochondrion	$L1_{33}$Lpa	4	0
	+acetate	1	0
	$L1_{33DD}$Lpa	2	0
	L133LpaDD	1	0
	$L1_{33}$KSA	4	4
	$L1_{33}$S236A	2	0
	+acetate	3	2

Table 2.1: Summary of attempted transfection of all lipoamidase constructs in this paper The only active lipoamidase that was successfully transfected into parasites was the attenuated S236A variant.

Primer name	Sequence
HA.F	GATTTATATAAGGAAAACTAGAAAATACCCATACGACGTCCCAGACTACGCTTGATAAGTCGACCTGCAG
HA.R	CTGCAGGTCGACTTATCAAGCGTAGTCTGGGACGTCGTATGGGTATTTTCTAGTTTTCCTTATATAAATC
$L1_{33}$BamHI.F	GGTGGTGGATCCATGAAACGAATATTCAGGTTGGT
$L1_{33}$BamHI.R	GGTGGTGGATCCATGAATATTCTGATTATTAGATACTAAT
ACP_{55}BamHI.F	GGTGGTGGATCCATGAAGATCTTATTACTTTGTATAATTTTTC
ACP_{55}BamHI.R	GGTGGTGGATCCTGGGTTTTTATTTTTTATCAAATTGTAATC
K159A.F	GGTGTGCCGCTCTTACTAGCAGGGTTAGGACAATCCTTG
K159A.R	CAAGGATTGTCCTAACCCTGCTAGTAAGAGCGGCACACC
S259A.F	GAAGTGATGCTGGTGGCGCTATCCGCATCCCTGC
S259A.R	GCAGGGATGCGGATAGCGCCACCAGCATCACTTC
HAAflII.R	GGTGGTCTTAAGTCAAGCGTAGTCTGGGACGTCGTATGGG
LpaAvrII.F	GGTGGTCCTAGGATGTTGGCACAAGAAAGTATACTAG
ACP_{55}AvrII.F	GTGGTCCTAGGATGAAACGAATATTCAGGTTGGTAAGA
$L1_{33}$AvrII.F	GGTGGTCCTAGGATGAAGATCTTATTACTTTGTATAATTTTTC
InsKpnI.F	TGGTATTAGTATCTAATAATCAGAATATTCATGGTACCATGTTGGCACAAGAAAGTATACTAG
InsKpnI.R	CTAGTATACTTTCTTGTGCCAACATGGTACCATGAATATTCTGATTATTAGATACTAATACCA
FKBPKpnI.F	GGTGGTGGTACCATGGGAGTGCAGGTGGAAACCAT
FKBPKpnIR	GGTGGTGGTACCTTCCGGTTTTAGAAGCTCCACATC
S236A.F	GAATCCTAACCATTATTCAGGTGGTTCTgCAGGCGGAGCGGGTGCTAG
S236A.R	CTAGCACCCGCTCCGCCTGCAGAACCACCTGAATAATGGTTAGGATTC
S236C.F	GAATCCTAACCATTATTCAGGTGGTTCTTgtGGCGGAGCGGGTGCTAGTG
S236C.R	CACTAGCACCCGCTCCGCCACAAGAACCACCTGAATAATGGTTAGGATTC
S236G.F	GAATCCTAACCATTATTCAGGTGGTTCTggAGGCGGAGCGGGTGCTAGTG
S236G.R	CACTAGCACCCGCTCCGCCTCCAGAACCACCTGAATAATGGTTAGGATTC
W210F.F	GGTCAAACGAATTATCCAGAATTAGGTTttAAGAATATTTCAGATTCAAAGTTATATGGCG
W210F.R	CGCCATATAACTTTGAATCTGAAATATTCTTAAAACCTAATTCTGGATAATTCGTTTGACC
A256G.F	GTTCCCATTGCTTCTGGAAGTGATGgTGGTGGCTCTATCCGCATCCCT
A256G.R	AGGGATGCGGATAGAGCCACCACCATCACTTCCAGAAGCAATGGGAAC
Y375F.F	GTTGATGGGGAACGTCTAATGAAAAATTtTTATACTGTAGCTGCTGGCTCAGC
Y375F.R	GCTGAGCCAGCAGCTACAGTATAAAAATTTTTCATTAGACGTTCCCCATCAAC
CG6.F	ATATAATATTCTTAACATCCTGTGAAGTTACCCAGGATCC
P4	GATAGCGATTTTTTTTACTGTCTG
P3.pLN	GCACAGATGCGTAAGGAGAAAATACC
P4.CG6	GCGCAATTAACCCTCACTAAAGGG
BsHprot.BamHI.F	TTTCAGGGC GGATCC ATGAGCATACCAAAAGATTTGCGTT
BsHprot.SalI.R	GCCGCTCGA GTCGAC TTAGTCTTCTTGTGTCATCTCTTCG

Table 2.2: Primers used to generate constructs and confirm integration.

Mutation	Relative Catalysis
Y375F, W210F, A256G	Approximately WT
S236C faster than S236G	About 10X slower than WT
S236A	At least 100X slower than WT

Table 2.3: Coarse assessment of lipoamidase mutants. Using the same assay in Supplementary Figure 2B, all lipoamidase mutants were tested against WT lipoamidase activity. Protein levels were made equivalent by western blotting, and multiple ten-fold dilutions were made of all lipoamidase variants to approximate relative catalysis. Mutations at S236 appear to have been the only mutations that imparted a significantly lower catalytic rate, with the S236A mutations resulting in the slowest catalysis.

2.9 Figures

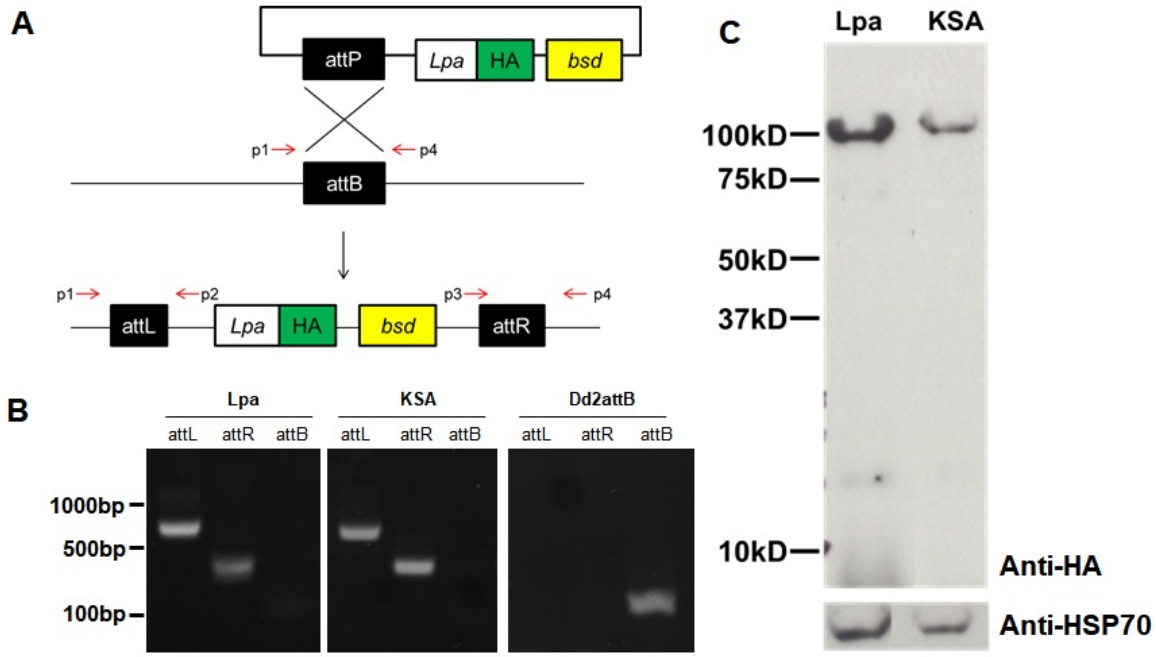

Figure 2.1: Targeting Lpa and KSA to the parasite cytosol

A. Model of genomic integration of the plasmid expressing lipoamidase into the dd2^{attB} genome. Co-transfection with the pINT plasmid provides the integrase that induces attB/attP integration. Primer pairs (red) for retention of the parental attB locus or the recombined attL and attR loci. **B.** PCR demonstrating genomic integration of the Lpa and KSA constructs. Both transgenic lines demonstrate 5′ (attL) and 3′ (attR) integration and the absence of the parental attB region. The parental dd2^{attB} line serves as a control. **C.** Western blotting to confirm lipoamidase expression. Antibodies specific for the HA tag were used to demonstrate expression of both the Lpa and KSA constructs in lysates of the transgenic parasites. *Pf*HSP70 is used as a loading control.

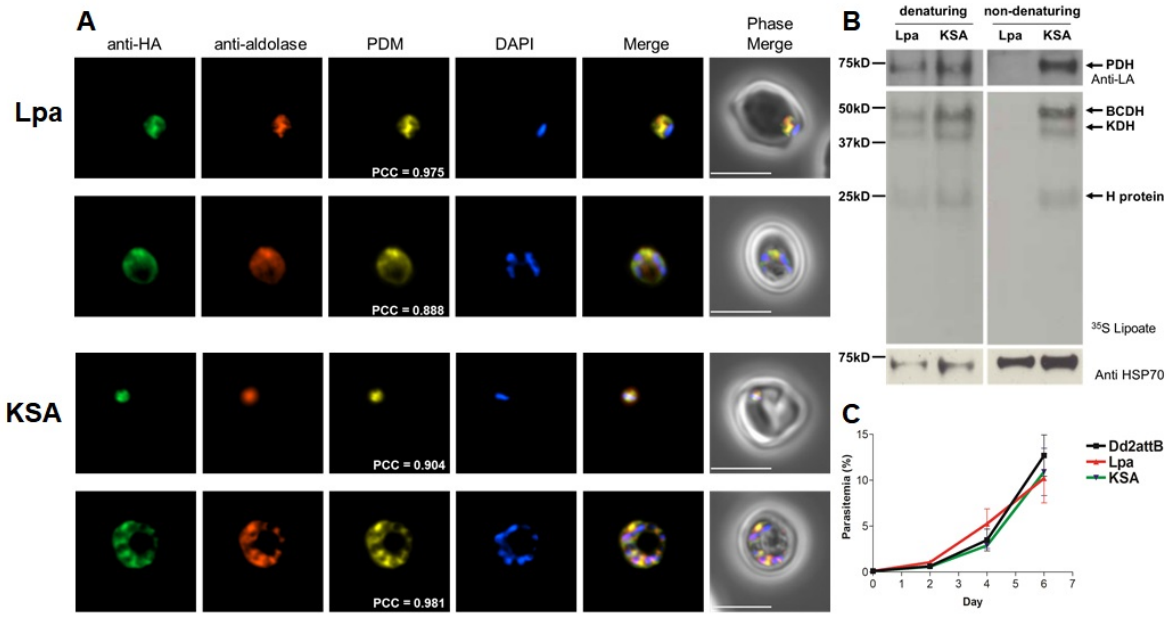

Figure 2.2: Lpa and KSA are expressed in the cytosol. A. Lpa and KSA are expressed in the cytosol. Immunofluorescence analysis of blood stage Lpa (top panels) and KSA (bottom panels) parasite lines showing co-localization of the lipoamidase constructs, labeled with an antibody specific for the hemagglutinin tag (anti-HA), with the cytosolic marker anti-Aldolase. The nuclei are labeled with DAPI. Scale bars = 5 microns. **B.** Lipoamidase is expressed in an enzymatically active form. In these multi-panel figures, anti-lipoate (anti-LA) western blot was used to identify lipoylated apicoplast PDH; autoradiograph of parasites treated with ^{35}S-lipoate (^{35}S-LA) was used to detect lipoylation of BCDH, KDH and H protein; and anti-*Pf*Hsp70 was used as a loading control. The left panels show results for parasites expressing Lpa and KSA that were lysed in SDS-PAGE loading buffer immediately prior to analysis, showing comparable protein lipoylation between both parasite lines. Under non-denaturing conditions (lysis in RIPA buffer followed by a 15 minute incubation at 30°C), cytosolic lipoamidase has access to organellar proteins and rapidly cleaves lipoate from all parasite proteins. **C.** Expression of either active or inactive lipoamidase imparts no growth phenotype. The growth of Lpa and KSA parasite lines was monitored by microscopy at two day intervals to demonstrate that these lines does not differ significantly from the parental dd2^{attB} parasite line. Error bars represent the SEM of three biological replicates.

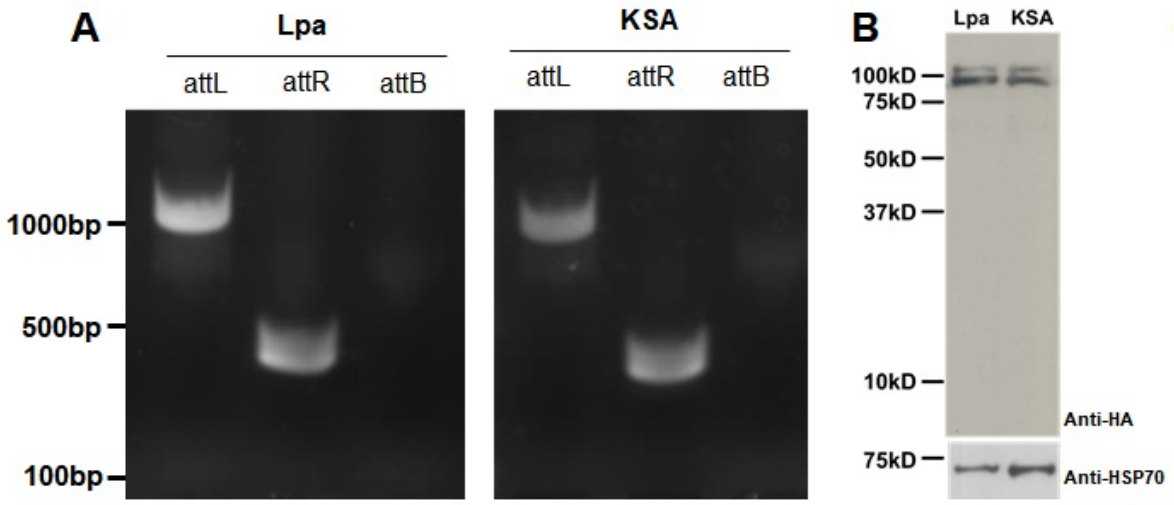

Figure 2.3: Targeting Lpa and KSA to the apicoplast. A. PCR demonstrates integration of apicoplast targeted Lpa and LpaKSA, and no evidence of parental attB locus. **B.** Western blotting for HA tagged proteins shows both Lpa and KSA as doublets, indicative of successful apicoplast trafficking.

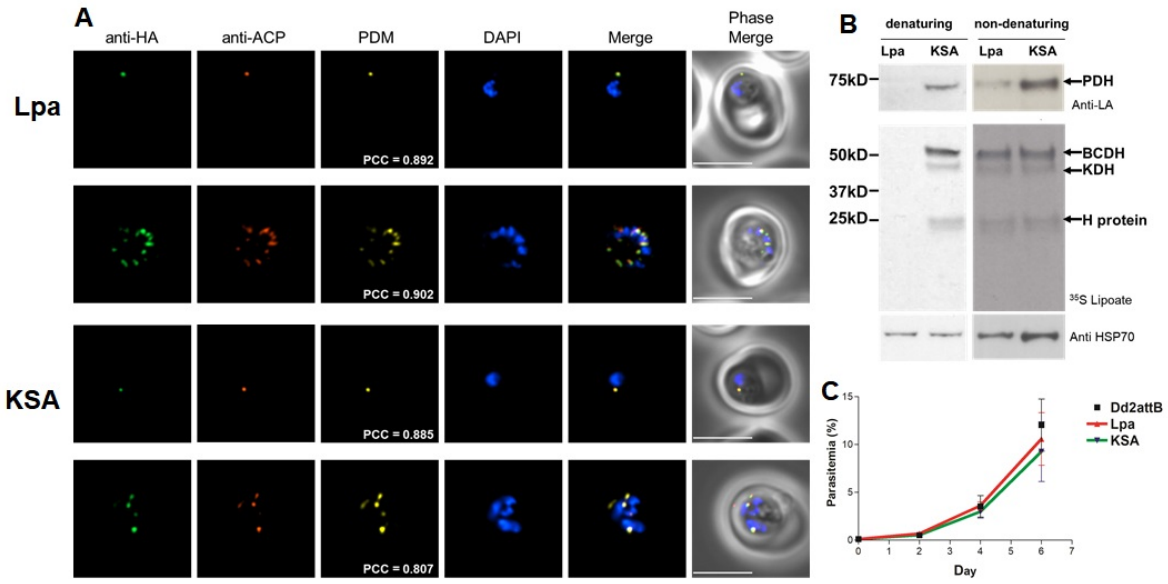

Figure 2.4: Lpa and KSA are efficiently trafficked to the apicoplast. A. IFA demonstrates efficient localization of Lpa constructs to the apicoplast. Fixation results in the apicoplast collapse. However, PDM and PCC values show strong colocalization values indicating both Lpa constructs are successfully expressed in the parasite apicoplast. **B.** Apicoplast targeted lipoamidase can only cleave the apicoplast PDH E2. Autoradiography shows that the mitochondrial lipoylation substrates are intact. Left panel shows complete cleavage of the PDH E2, suggesting that there is some in vivo prevention of full delipoylation. **C.** Expression of either active or inactive lipoamidase imparts no growth phenotype. Error bars represent the SEM of three biological replicates.

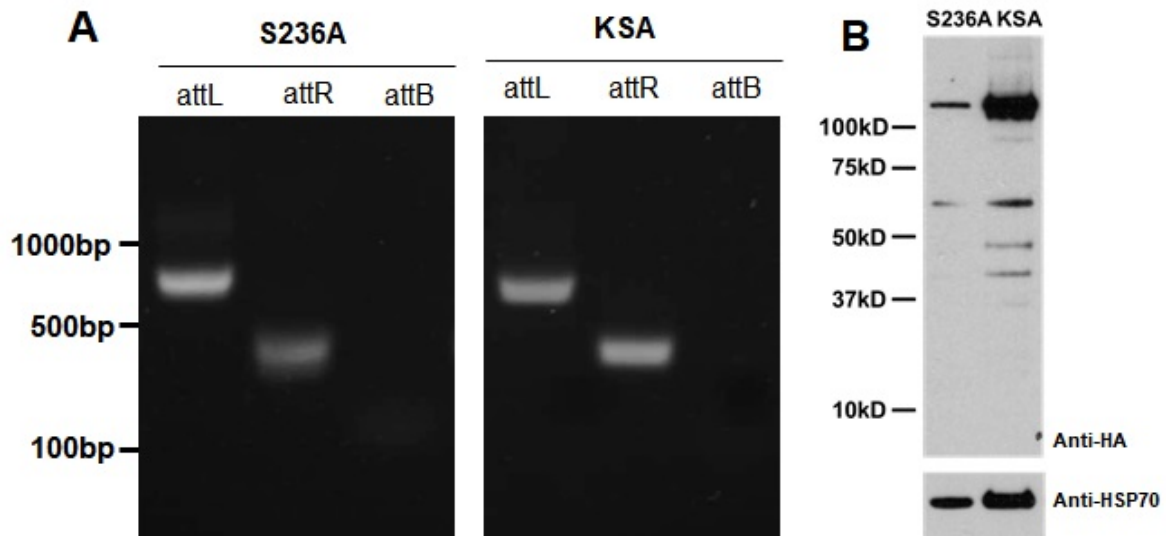

Figure 2.5: Targeting S236A and KSA to the mitochondrion. A. PCR shows genomic integration of both the S236A and KSA constructs targeted to the mitochondrion. **B.** S236A expression is strongly attenuated. Unlike the abovementioned constructs, the active, attenuated form of lipoamidase is somehow expressed at much lower levels than KSA. Both lanes also show degradation products, suggesting the parasiteâĂŹs intolerance of both constructs.

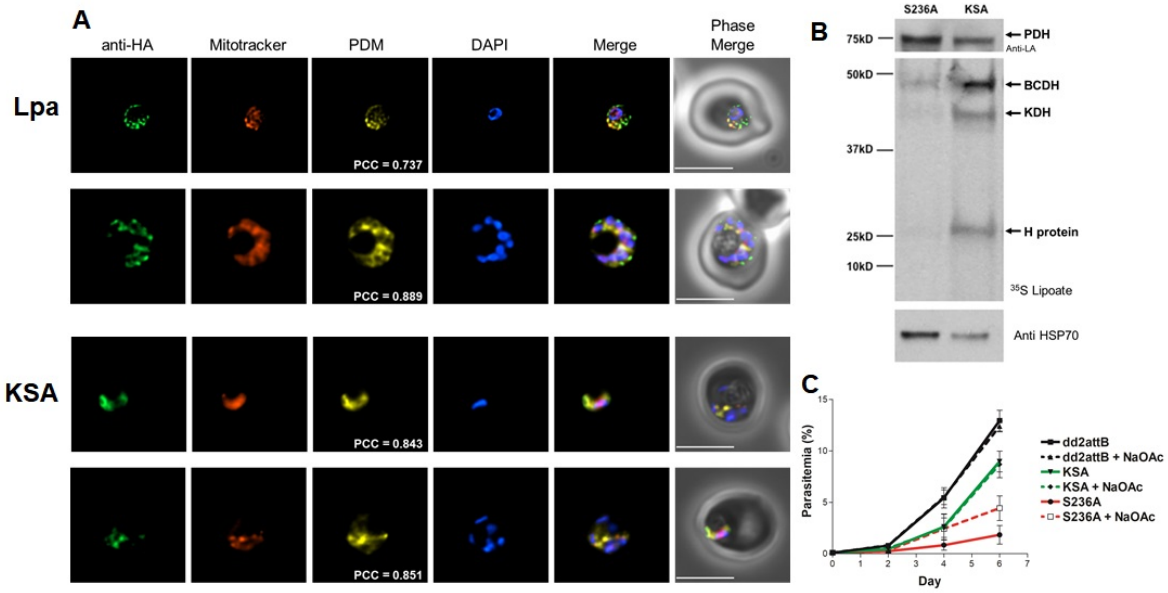

Figure 2.6: Mitochondrial S236A expression in the mitochondrion imparts a growth defect. A. IFA confirms mitochondrial expression of both lipoamidase variants. **B.** Mitochondrial lipoamidase can cleave lipoate from mitochondrial substrates. Densitometry analysis shows at least an 80% reduction of lipoylation of all the mitochondrial substrates when normalized against the loading control. Apicoplast PDH E2 lipoylation remains the same between the attenuated and inactive forms of lipoamidase. **C.** Mitochondrial expression of lipoamidase imparts a growth phenotype. S236A expression imparts the most drastic phenotype, while a modest defect appears in KSA expressing lines. Dotted lines represent cultures grown in 5mM sodium acetate. Error bars represent SEM with biological triplicates.

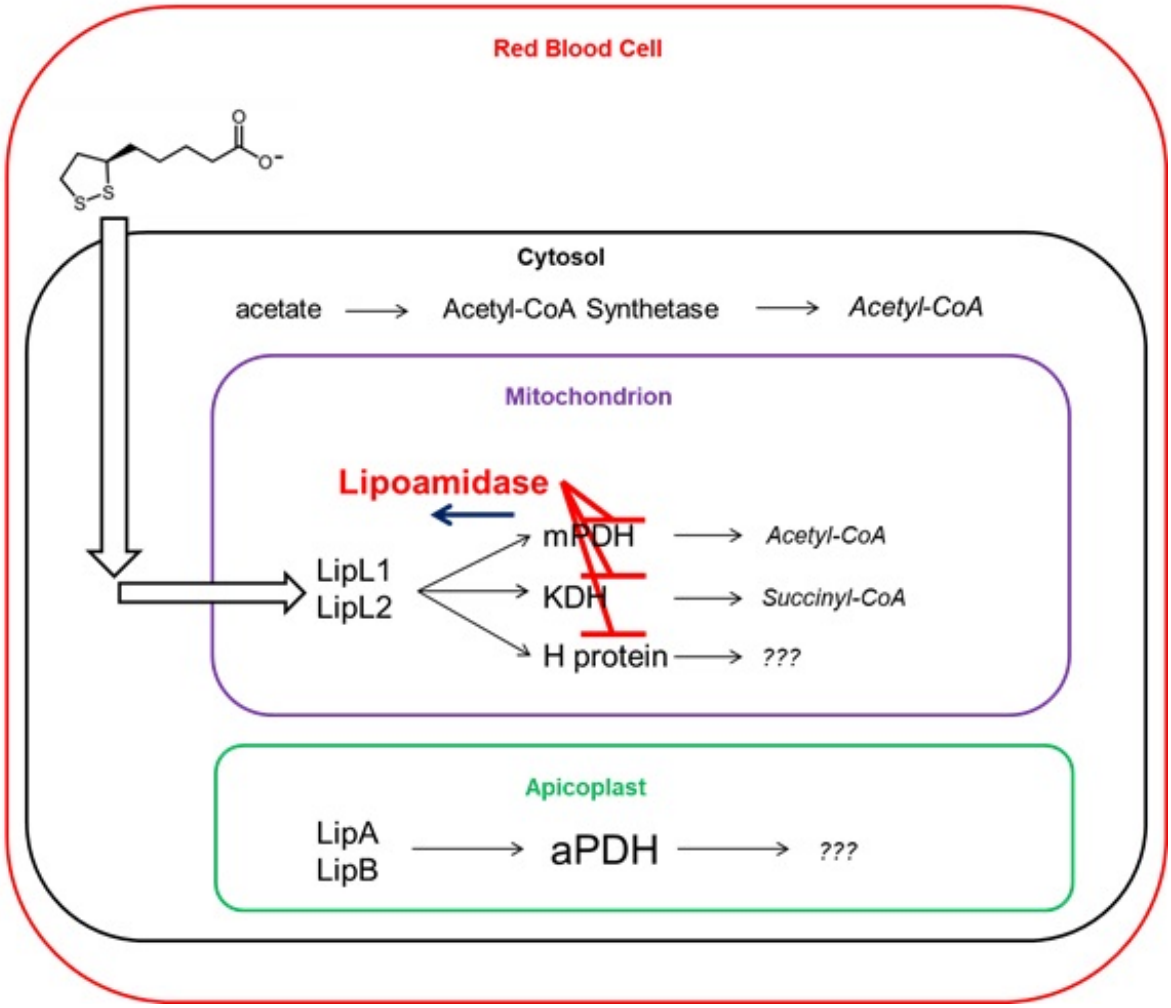

Figure 2.7: Model of parasite lipoylation and lipoamidase activity. Active lipoamidase expression in the mitochondrion imparts a growth phenotype. The ability of acetate to rescue this phenotype suggests that the generation of acetyl-CoA by a mitochondrial PDH, likely the gene formerly annotated as a BCDH. It remains a possibility that some of the growth deficit (and possibly the deficit in the KSA expressing parasites) is due to abrogation of H protein activity.

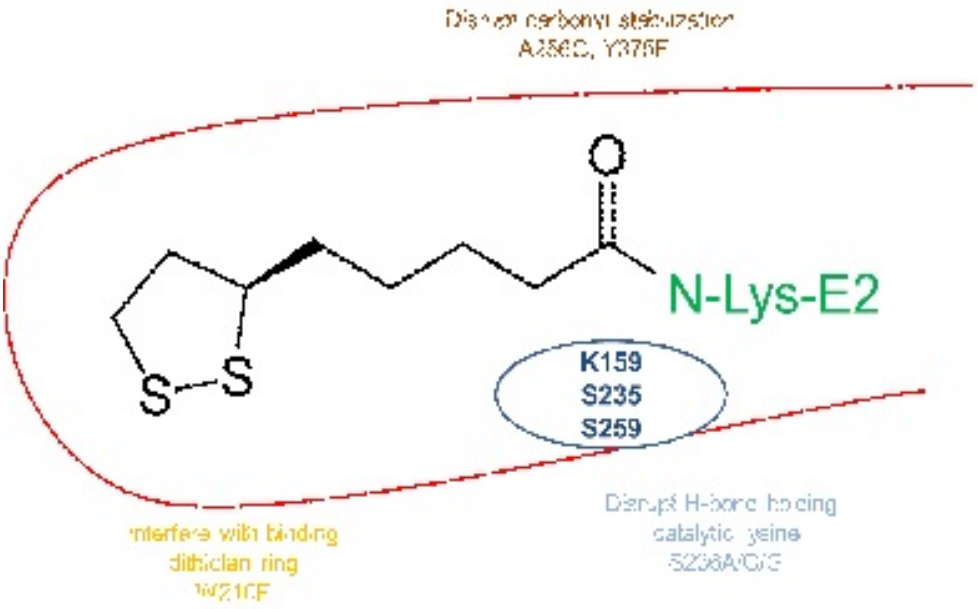

Figure 2.8: Cartoon depiction of lipoamidase mutagenesis rationale. Mutations at the described positions were made to putatively disrupt lipoamidase's ability to efficiently bind a lipoylated substrate. These mutations were thought to disrupt carbonyl stabilization of the lipoamide bond, disrupt hydrogen bonding of the catalytic lysine (K159), and interfere with hydrophobic interaction at the dithiolan ring. The K159, S235 and S259 residues make lipoamidase's catalytic triad.

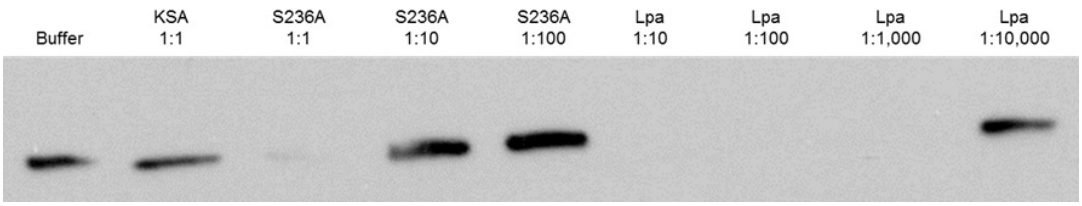

Figure 2.9: Attenuation of the S236A lipoamidase mutant. Delipoylation assay to show S236A is at least 100 times less catalytically efficient when compared to wild type lipoamidase. Concentration of Lpa variants were normalized based on HA blotting of cell lysates, and dilutions were made for assay. First lane shows benchmark lipoylation signal of purified and lipoylated H protein from *Bacillus subtilis*.

Chapter 3

Mitochondrial lipoylation may be essential in *Plasmodium berghei*

3.1 Abstract

Plasmodium berghei shares *P. falciparum's* dual lipoylation strategy - a biosynthetic pathway in the apicoplast and a scavenging pathway in the mitochondrion. The apicoplast resident lipoylation machinery and substrates are dispensable in the blood stages of both parasites, while mitochondrial lipoylation may be essential. There are three mitochondrial proteins that require lipoylation for function: the BCDH, the KDH and the H protein. These proteins are also likely to require a shared protein, the mitochondrial dihydrolipoamide dehydrogenase (E3), in order to recycle lipoate for catalysis. Knockout experiments for all four of these gene products were attempted in *P. berghei*, resulting in complete dispensability of the KDH lipoylated subunit (E2) in the blood stage of the parasite, and BCDH E2 and the E3 recycling subunit. Multiple attempts to knockout the H protein failed, suggesting an essential, noncanonical role for this protein.

3.2 Introduction

Lipoic acid is a small fatty acid with two sulfurs at the end of the aliphatic chain. It serves as a cofactor in several, crucial biochemical pathways in life, most extensively described in the pyruvate dehydrogenase reaction, where pyruvate is used to produce acetyl-CoA, bridging glycolysis to the citric acid cycle (Perham, 2000). Lipoate-mediated catalysis can be quite complicated, involving multiple other cofactors and large protein complexes (Introduction chapter), and the ability to perform lipoate-mediated catalysis appears to be crucial for most forms of life. Gene deletions in lipoate synthesis and/or ligation pathways result in severe growth phenotypes or lethality in E. coli (Cronan et al., 2005), Arabidopsis (Ewald et al., 2014), yeast (Schonauer et al., 2009), mice (Yi and Maeda, 2005) and humans (Baker et al., 2014; Mayr et al., 2014; Smith et al., 2012).

Plasmodium harbors a lipoate biosynthetic pathway in its apicoplast in order to lipoylate the apicoplast resident pyruvate dehydrogenase (PDH). Knockouts experiments of the apicoplast lipoylation machinery and PDH in multiple species of *Plasmodium* result in no defect in blood stage development, but do demonstrate growth phenotypes outside the blood stage, demonstrating importance of a lipoylated and fully functional PDH in the mosquito (Cobbold et al., 2013) or liver stages of parasite development (Falkard et al., 2013; Pei et al., 2010), but not in the blood stages (Günther et al., 2007; Laine et al., 2015).

The mitochondrion of the parasite houses two enzymes with homology to lipoate ligases, LipL1 and LipL2, that use scavenged lipoate to lipoylate

three mitochondrial substrates (Afanador et al., 2014, 2017; Allary et al., 2007). These three mitochondrial enzyme complexes are the branched chain alpha-ketoacid dehydrogenase (BCDH), the alpha-ketoglutarate dehydrogenase (KDH) and the H protein of the glycine cleavage system (Allary et al., 2007). The BCDH and KDH complexes are composed of three enzymes that perform a complicated, three step catalysis (**Figure 3.1**). The E1 subunit decarboxylates the substrate, the E2 subunit, which is the lipoylated protein, performs lipoate-dependent ligation of the substrate to coenzyme A, and the shared mitochondrial dihydrolipoamide dehydrogenase (the E3 subunit) recycles the lipoate cofactor by reoxidizing lipoate. The glycine cleavage system generates 5,10-methylenetetrahydrofolate for single carbon chemistry using a mechanism similar to the BCDH and KDH. The P protein catalyzes the decarboxylation of glycine, the T protein and the lipoylated H protein work together to transfer to alpha-carbon of glycine to tetrahydrofolate and release ammonia, and the L protein recycles the lipoate cofactor to the oxidized state.

P. berghei scavenges lipoate for mitochondrial lipoylation, and interference with lipoylation inhibits parasite growth (Falkard et al., 2013; Wang et al., 2017), suggesting that mitochondrial lipoylation may be essential to the murine parasite as it appears to be in *P. falciparum* (Allary et al., 2007). Lipoate analogs have demonstrated *in vivo* activity (Zachar et al., 2011) and are currently in clinical trials for cancer therapies, raising the possibility that lipoate metabolism could be targeted in malaria parasites (Alistar et al., 2017; Dörsam and Fahrer, 2016; Lamar et al., 2016; Pardee et al., 2014; Stuart et al., 2014; Thomsen-Zieger et al., 2003)

Work demonstrated in Chapter 2 and in the knockout of the E1 alpha subunit of the BCDH in *P. berghei* (Oppenheim et al., 2014) demonstrates that the BCDH is likely misannotated and really doesn't have BCDH activity. This hypothesis is consistent with the apparent lack of many genes in the genomes of malaria parasites that would be needed downstream of a BCDH enzyme to further metabolize BCDH products. Instead, the BCDH appears to be a mitochondrial pyruvate dehydrogenase, forming acetyl-CoA from from pyruvate. Metabolomics data in *P. falciparum* points to the BCDH being the chief source of glucose-derived acetyl-CoA (Cobbold et al., 2013), suggesting the enzyme complex could be essential in light of the fundamental roles of acetyl-CoA in cell biology. The KDH E1 subunit has been knocked out in *P. falciparum* (Ke et al., 2015), and the lipoylated E2 appears to be disruptible in the work performed in Chapter 3, making knockout of the entire KDH dispensable in the blood stage of the parasite. Finally, it is hard to predict whether the H protein is essential since it is very unlikely to be functioning as part of a glycine cleavage system do to the lack of appropriate P protein and T protein candidates in the genomes of malaria parasites. The gene knockout experiments sought to answer the question of the essentiality of these three proteins in *P. berghei*. As an additional approach, two variants of lipoamidase were targeted to the mitochondrion of the parasite to confirm that lipoylation of these substrates were important for parasite survival as they were in *P. falciparum*.

3.3 Results

3.3.1 Lipoamidase expression is tolerated in *P. berghei*

Based on of success of lipoamidase expression in *P. falciparum* described in Chapter 2, expression in *P. berghei* was also attempted. The attenuated S236A and inactive KSA variants of lipoamidase were the first candidates to test due to their low activity. The mitochondrial targeting sequence of the first 33 amino acids from the *P. falciparum* LipL1 was retained in this experiment due to the high sequence similarity to the leader sequence in the *P. berghei* ortholog of LipL1. Sequences were cloned into the pL1694-mCherry knockin expression vector, (**Figure 3.2**), to insert lipoamidase driven by the *Pb*Hsp70 promoter into the dispensable 230p locus for potential interrogation of the entire lifecycle (Janse et al., 2006). The gene product would be fused to the mCherry fluorescent protein in order to attempt to use live microscopy for co-localization assays. Both transfections were successful by PCR (**Figure 3.3**). The KSA variant was successfully single cloned (**Figure 3.4**), and western blot analysis of parasite lysate confirmed that the KSA-mCherry variant was expressed (**Figure 3.5**). Microscopy experiments to confirm mitochondrial expression were inconclusive and require further experimental troubleshooting due to poor mCherry fluorescence in live microscopy.

3.3.2 KDH E2 is dispensable in *P. berghei*

Initial transfections with standard pDEF knockout constructs failed, but transfection and generation of a clone of the knockout was successful using

a construct from the PlasmoGem project (Schwach et al., 2015), a library of highly efficient transfection constructs that employ very large homology arms for efficient homologous recombination (**Figure 3.6A, B, Table 3.4**). As there was no obvious growth phenotype observed in a clone of Δkdh parasites, the drug cassette was recycled successfully using 5-fluorocytosine treatment and successfully single-cloned (**Figure 3.6C**). This procedure would allow us to make additional genetic modifications with plasmids containing the hDHFR drug resistance cassette, including other gene deletions.

3.3.3 BCDH, H protein and the shared E3 subunit may be important for parasite growth

We attempted to knockout the BCDH E2 protein using the same approach described above for the KDH E2. We were able to detect successful Δbcdh parasites by diagnostic PCR (**Figure 3.7**), but multiple single cloning attempts resulted in wild type parasites (**Figure 3.8**). This suggests that the proportion of knockout parasites to wild type parasites in the post-transfection population was low or that wild type parasites have a significant growth advantage compared to the knockout parasites. An initial attempt at determining the proportion of wild type to knockout parasites yielded substandard results (**Table 3.5**). If the data are correct, the proportion of mutant parasites would be approximately 1/8th the total parasite population, but extrapolating from these data strongly suggests that the number of mice needed to obtain at least one clonal population of the knockout was too high to be feasible. Multiple attempts were made to increase the proportion of Δbcdh parasites, including phenylhydrazine treatment, *ex vivo* culturing with acetate supplementation

and high-dose pyrimethamine treatment (**Figure 3.9)**, and constructing a fluorescent tag for enrichment by flow cytometry (**Table 3.1**). We were not able to clone Δbcdh parasites using these methods and it is likely that this line has a significant fitness defect compared to wild type parasites.

Attempts to knock out the H protein by both the pDEF and Plasmogem constructs failed multiple times, but parasites never came up, so it is unclear if the knockout is not tolerated or if all transfections failed. Knockout out the mitochondrial E3 subunits resulted in parasites that were positive for the disrupted locus (**Figure 3.10**).

3.4 Discussion

3.4.1 Lipoamidase in *P. berghei*

Further work in exploiting lipoamidase as a metabolic probe in *P. berghei* would be informative on interrogating the importance of lipoylation in different life cycle stages of the murine parasite. Current experiments have not confirmed mitochondrial targeting of the protein - this is partially due to the constraints of our system. The gene was cloned with an mCherry fusion protein; however, the intrinsic fluorescence of this line is too low to be useful in live fluorescence microscopy experiments. Immunofluorescence experiments are complicated by needing a co-localization marker for the mitochondrion that is compatible with fixation methods. The only easily available marker for mitochondrial localization Mitotracker Green, does not work well with aldehyde based fixation methods. One group appears to have been successful in fixing blood stage parasites with methanol for Mitotracker Green staining

(Boysen and Matuschewski, 2011), but this method was not successful as yet in our experiments. However, the banding pattern observed in anti-mCherry western blot is similar to the banding pattern observed in the anti-HA blot used in the successful *P. falciparum* lipoamidase experiments (Chapter 2). These similarities are possibly suggestive of mitochondrial targeting, but confirmation may require attaching a different tag and/or optimizing microscopy techniques.

In *P. falciparum*, acetate supplementation was critical for the success of the lipoamidase experiments. This technique is difficult to employ with *P. berghei* due to the vagaries of mouse metabolism. The growth defect imparted by mitochondrial lipoamidase expression was rescued by treatment with 5 mM acetate supplementation, but it is unlikely that this is the lowest amount of acetate needed to rescue growth appreciably. Running a parallel experiment in a mouse would be complicated because mouse acetate levels appear to be tightly regulated round 80 μM, with acetate supplementation clearing serum in under one hour (Frost et al., 2014). Considering these constraints, attempting to observe acetate rescue in a parasite line expressing lipoamidase in a mouse may not be feasible. On the other hand, considering the tightly regulated acetate concentration in mouse blood and the ability for parasites to infect metabolically rich reticulocytes (Srivastava et al., 2015), it may be possible to have a parasite line that can express the attenuated lipoamidase, and another parasite line that can express the fully active lipoamidase, a phenomenon that appears impossible in *P. falciparum*. Performing a radiolabeling experiment as in Chapter 2 with radiolabeled lipoate to observe reduced lipoylation would

have to be performed in *ex vivo* culture in order to prevent loss of the radiolabel in unrelated tissues. There may also be complications associated with host cells like white blood cells and immature reticulocytes, which could retain some lipoylated proteins. Despite these drawbacks, exploring lipoamidase activity in the murine model system provides an opportunity to observe parasite growth under *in vivo* conditions more relevant to the human disease state. In particular, the murine model makes it possible to interrogate the significance of protein lipoylation in different malaria lifecycle stages.

3.4.2 Disruption of canonical lipoate-mediated biochemistry in the parasite mitochondrion

The ease with which the KDH E2 knockout was obtained and cloned in *P. berghei* suggests that the enzyme is completely dispensable in the blood stage of parasite development, even for parasites maintained *in vivo*. This is consistent with the observation that the KDH E1 can be deleted in *P. falciparum* (Ke et al., 2015). The simplest explanation of these results is that the KDH proteins are indeed components of a KDH complex and don't have any other important roles outside of TCA cycle metabolism. Ke and coworkers showed that the *P. falciparum* KDH E1 knockout line could not complete mosquito stage development and we would assume that our *P. berghei* E2 knockout would also fail to thrive during these stages.

Our inability to isolate a BCDH E2 knockout is strongly indicative of a severe growth phenotype. Knockout of the E1alpha subunit of the BCDH imparts a severe growth phenotype (Oppenheim et al., 2014) that required immunocompromised mice for single cloning experiments. Although this

approach has several drawbacks, it may also be necessary for our BCDH E2 knockout. It is possible that the growth phenotype is so severe that parasites successfully diluted to one parasite per mouse during cloning experiments won't tend to result in infected mice. This scenario would require a different means of cloning, e.g. loading more than one parasite per mouse in order to achieve successful infections. Finally, the qPCR experiments are highly problematic - it was impossible to perform this experiment without getting signal from non-template controls, so whatever extrapolation that can be made from the data would be an overestimation of the percentage of knockouts vs. wild types. It is also likely that over time the wild type population would continue to expand into a larger and larger proportion of the population, and continuous passage of this mixed population would result in a vanishing population of knockouts. All these effects together would make single cloning using standard methods close to impossible.

Our transfection experiments in *P. berghei* suggest that the BCDH and H protein are important for blood stage parasite replication. It is likely that the BCDH E2 knockout would behave similarly to the BCDH E1 knockout *P. berghei* and replicate slowly with the limitation of only being able to use reticulocytes as host cells (Oppenheim et al., 2014). It is likely that the *P. berghei* BCDH complex is actually a pyruvate dehydrogenase (PDH) and not a branched chain alpha-ketoacid dehydrogenase, an observation made in Chapter 2 and in metabolomics work (Cobbold et al., 2013; Oppenheim et al., 2014).

The experiments here help clarify thiamine dependence of blood stage

parasites. Both blood stage *P. falciparum* and *P. berghei* are dependent on exogenous thiamine (vitamin B1) (Hellgren et al., 2017; Wrenger et al., 2006), and treatment of both parasites with a toxic analog, oxythiamine, results in reduced parasite growth. The only identified enzymes in the parasite that appear to be thiamine dependent are the apicoplast PDH, KDH, the BCDH and transketolase (Chan et al., 2013). Since the KDH and PDH seem to be dispensable in the blood stage, the BCDH and transketolase could be the important thiamine-dependent enzymes inhibited by oxythiamine. This line of reasoning is consistent with the hypotheses that the parasite's thiamine utilization pathway (Wrenger et al., 2006) and pentose phosphate shunt (Hasan et al., 2015; Preuss et al., 2012) are potential drug targets. Treatment of the BCDH E1 knockout or a BCDH/KDH double knockout should be able to confirm this, unless the BCDH/KDH knockout alone is lethal.

3.4.3 Evidence of novel lipoate dependent biochemistry in the mitochondrion

It is unclear whether the BCDH is the sole producer of acetyl-CoA in the mitochondrion of malaria parasites. The *P. falciparum* KDH E1 subunit can catalyze both pyruvate and alpha-ketoglutarate at sufficiently comparable rates (Chan et al., 2013), suggesting that the *P. falciparum* KDH can function as a backup PDH, and this catalytic setup may be mimicked in *P. berghei*. As such, it may be that knockout of the BCDH E1 or E2 subunits survive due to some functional redundancy with the KDH, and that a double knockout would be synthetically lethal due to the complete inability to generate acetyl-CoA from pyruvate in the mitochondrion.

It has been recently shown that *Staphylococcus aureus* is able to secrete its lipoylated PDH, and that this protein can serve to strongly attenuate the macrophage activation via TLR2-dependent manner, allowing for greater survival in mice (Grayczyk et al., 2017). It is unclear whether or not Plasmodium can secrete lipoylated proteins, but this may explain potential differences in the different permittivity of knockouts between *P. falciparum* and *P. berghei*. It would be interesting to observe the immunological outcomes of *P. berghei* knockouts of the lipoylated proteins with "rescue" by IV injected, purified and lipoylated proteins as performed in *S. aureus* to see if secretion of lipoylated proteins is a conserved strategy among pathogens. Ultimately, the potential metabolic differences and the nature of the mouse model vs. tissue culture require a good deal of rigorous interrogation to fully flesh out the role of the BCDH in both species.

It was a surprise to us that the mitochondrial E3 subunit appears to be disruptible. This protein should be important for the activity of the KDH, BCDH and the H protein, and deleting it should be equivalent to deleting all three lipoylated proteins. Since the BCDH and the H proteins appear independently to be important for parasite growth, it seems unlikely that the E3 subunit could be deleted. Although the experiments performed here are inconclusive, the apparent ability to knockout this gene raises the possibility that alternatives pathways may be at work in the parasite. Either another protein can catalyze the oxidation of lipoate instead of the E3 subunit, or conditions in the mitochondrion facilitate this process. This scenario is not hard to imagine since the glutathione redox potential in the mitochondria

of blood stage *P. falciparum* parasites is -328 mV (Mohring et al., 2017), a value similar to that of lipoate, suggesting potential spontaneous reduction of oxidized lipoate.

The H protein remains a mystery in parasite biology. The H protein is part of the glycine cleavage system, which is canonically comprised of four proteins, including the H protein, but the parasite does not code two of the four proteins in its genome, and knockout of the only other remaining possible member of the GCV, the T protein, has no apparent consequence throughout the parasite life cycle (Varadarajan et al., 2014). In addition, blood stage parasites also appear unable to perform glycine cleavage (Varadarajan et al., 2014), so the H protein is probably maintained for some other purpose. One explanation is that the H protein has a moonlighting function. One such function is to participate in lipoylation reactions by serving as the source of lipoate for lipoyl transferases that then lipoylate other proteins (Cao et al., 2018) . Experiments performed with pure recombinant proteins show that the *P. falciparum* H protein does not play this role in conjunction with other lipoate attachment enzymes (Afanador et al., 2014).

One hypothesis for the role of the H protein involves oxidative defense. Work in *E. coli* shows that the shared E3 subunit can act in concert with lipoylated proteins to mitigate redox damage (Feeney et al., 2011), and work performed in our lab (Afanador et al., 2014) demonstrates redox sensitivity by the *Plasmodium* mitochondrial lipoate ligase, suggesting a possible lipoate-based redox response in the parasite. Interestingly, we rarely see a band for lipoylated H protein in anti-lipoate western blots of *P. falciparum* and *P.*

berghei parasites. This might suggest that the H protein is not lipoylated; however, lipoylated H protein is always detected at levels similar to other lipoylated proteins in autoradiographs of parasites treated with ^{35}S-lipoate. This difference in detection might be due to chemical modifications that result from scavenging reduced oxygen species. Anti-lipoate antibodies do not recognize lipoate with oxidized sulfur atoms such as sulfenic acid (SOH), sulfinic acid (SO_2H), or sulfonic acid (SO_3H). Future studies will tease out whether this hypothesis has merit. Since the S236A lipoamidase line reduces H protein lipoylation, these parasites might be more sensitive to different forms of oxidative stress. Alternatively, new knock down approaches could be used to specifically reduce H protein levels, changing the sensitivity of these parasites to oxidative stress in a reversible manner. Ultimately, due the lack of understanding of how a lipoylated H protein functions outside of the glycine cleavage system, determining the role of the H protein may remain a mystery for some time.

3.4.4 Methods Improvement

The difficulty in obtaining single clones of the BCDH KO highlights some of the problems in dealing with genetic manipulation in *P. berghei*. The pyrimethamine/hDHFR selection cassette demonstrates strong repression of wild type parasites (Janse et al., 2006; Philip et al.), but inconsistent clearance makes post-transfection wild type populations unavoidable. Also encompassed here is the issue of transfection efficiency: even with the added 10-fold improvement in transfection efficiency of the PlasmoGem constructs

against standard plasmids (Pfander et al., 2011), the theoretical maximum efficiency of successful transfectants is only 10%. For genetic modifications that result in a growth phenotype, that genotype will be seriously outnumbered by the pyrimethamine-suppressed wild type population waiting to rebound. Novel methods would be required to successfully isolate parasites with fitness defects resulting in slow growth. Some attempts to enrich knockout parasites were made in this project without success. Other methods have been used by other groups, e.g. flow sorting to enrich for modified parasites (Kenthirapalan et al., 2012) and coupling short-term *ex vivo* culturing with mouse-incompatible drugs (Jambou et al., 2011; Soga et al., 2017). It is not clear whether these methods would work to enrich the Δbcdh line or other lines with significant growth defects.

3.5 Future Directions

3.5.1 Lipoamidase in *P.berghei*

Bringing in both the apicoplast and mitochondrially targeted lipoamidase variants used in *P.falciparum* in Chapter 2 would help to resolve the importance of organelle specific lipoylation in those two compartments due to the apparent dual organelle activity of the *P.berghei* lipoylation enzymes (Falkard et al., 2013; Wang et al., 2017). As in the *P.falciparum* experiments, localization experiments, growth curves and Western blotting/autoradiography can be performed to determine the importance of organelle specific lipoylation. It would also be interesting to see if any reticulocyte preferences occur as an alternate metabolic phenotype to the growth deficit observed in the *P.falciparum*

experiments, as there are no reticulocytes in *P.falciparum* culture. The mCherry marker should be swapped for a different tag because of its poor visualization; one attractive option is a newly described red fluorescent protein (mScarlet) that is superior to mCherry (Bindels et al., 2017), but is likely still compatible with mCherry antibodies. This would bring an upgraded tool to the field that should be compatible with current methods.

Continuing an apicoplast targeted lipoamidase line outside the blood stage may confirm previously observed phenotypes of apicoplast lipoylation being important, but not essential, for liver-stage development (Falkard et al., 2013; Nagel et al., 2013). Continuing a mitochondrially targeted lipoamidase line outside the blood stage may impart a partial phenotype, potentially bypassing the pre-oocyst arrest of the BCDH E1 knockout (Oppenheim et al., 2014b). This may allow us to interrogate the importance of mitochondrial lipoylation from the oocyst to the liver stage, an observation that has not been done before, and may not be possible without a lipoamidase. Putting lipoamidase under a stage specific promoter (Lacroix et al., 2011; De Niz et al., 2015) may also help fine tune these kinds of experiments. First pass experiments to determine gross importance would include determining oocyst development, sporozoite numbers, and looking for delays to prepatency. If any phenotypes emerge, more granular experiments can be performed, including immunofluorescence assay for organelle specific lipoylation during sporozoite and intrahepatic stages, or lipoate analog poisoning during the liver stage to clarify if previous experiments that could not fully determine if biosynthesis or scavenging was being poisoned by lipoate analogs.

Ultimately, repeating the *P.falciparum* lipoamidase experiments in *P.berghei* would help clarify the mystery regarding organelle specific lipoylation, and would allow exploration of the importance of lipoylation in the non-red blood cell stages that could not be performed using other methods. This is especially likely considering the difficulty of the knockout experiments described below.

3.5.2 Knockouts of lipoylation enzymes and lipoylated substrates in *P.berghei*

Finishing the knockout experiments would likely take the most amount of time. The easiest to start with is the KDH E2 knockout. This genotype has already been cloned, and should be ready for growth assays and interrogation of non-blood stage phenotypes as described above. Because this line is markerless, it should also be ready for further genetic modifications.

The BCDH E2 knockout would likely need to be retried from scratch. The transfection cassette should be modified in order to accommodate flow sorting to help enrich for transfectants. Experiments can also try the blasticidin or puromycin resistance cassette for *ex vivo* selection (Soga et al., 2017) as they appear to work faster than pyrimethamine selection and should kill, rather than inhibit, wild type parasites. Transfection using the most recent AMAXA nucleofector is likely the best transfection strategy, as it affords an improvement in transfection efficiency compared to the AMAXA that the department currently has. This should help the starting population of transfectants get a further head start than the original transfections. Transfection and single cloning should still be performed in phenylhydrazine pre-treated mice for optimal conditions for the mutants. Once a clone is obtained, characterization

as described for the KDH can be performed.

Though an attempt can be made to clone the mitochondrial E3 knockout obtained, it may phenocopy the BCDH E2 knockout or be worse as it should have served as a triple knockout of the BCDH E2, KDH E2 and H protein. The mitochondrial lipoyl ligases can also be attempted as we have those constructs, though they can be expected to have phenotypes expected of a double knockout (LipL2) or triple knockout (LipL1).

As discussed previously, metabolic rescue would be difficult in any of these lines, assuming knowledge of the disrupted pathways. Pre-treatment with phenylhydrazine may be the best bet in order to improve reticulocyte numbers and provide a more nutrient rich environment for the mutant parasites. Pilot attempts at rescuing with increased glucose in the media in *P.falciparum* lipoamidase demonstrates no effect, so putting mice on a high sugar diet may not improve results. Supplementation may be possible with long term ex vivo culture (Jambou et al., 2011); this could be coupled to *ex vivo* drug selection for a further improved proportion of mutant parasites to wild type parasites.

Ultimately, it would take a good deal of work to fully flesh out the necessity of mitochondrial lipoylation in P.berghei because of the predicted severe growth phenotypes and the lack of rigorously tested methods outside of standard transfection and selection. Pursuing this project would require validation of these methods, making the ultimate paper an ambitious project.

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3.7 References

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3.8 Tables

Primer Name	Sequence	Function
Link.mCherry.For	TCTGGTGGAGGTGGAAGTGCTAGCTGGTGAGCAAGG GCG	Oligo to generate mCherry linker to hDHFR
mCherry.yFCU.Rev	ATTCCCCCTGTCACACTAGCCTTGTACAGCTCGTCCA TGC	Oligo to link mCherry to yFCU
mCherry.yFCU.Stop.Rev	ATTCCCCCTGTCACACTAGCTTACTTGTACAGCTCGT CCA	Oligo to link mCherry to stop before yFCU transcription
pL1694MCS.1	GATCGTCGACTCCGGAGGGCTCGAGACGCGTC	Generation of MCS between mCherry and POI in pL1694 plasmid
pL1694MCS.2	GATCGACGCGTCTCGAGCCCTCCGGAGTCGAC	Generation of MCS between mCherry and POI in pL1694 plasmid
BspEI.L133 For	GGTGGTTCCGGAATGAAACGAATATTCAGGTTGG	5' Amplification of LipL1 coding sequence and appending BspEI restriction site
XhoI.Lpa.Rev	GGTGGTCTCGAGTTTTCTAGTTTTCCTTATATAAATCG TT	3' Amplification of lipoamidase coding sequence and appending XhoI restriction site
PbHsp70.5UTR.F	CCCAAAACATATTTCTTTGTATTATATACATTTTTGC	Sequencing primer for Hsp70 promoter
RFP.rev	GCCGTTCACGGAGCCCTC	Sequencing primer for gene upstream of mCherry
Pb230p5.For	GCAAAGTGAAGTTCAAATATGTG	5' integration for 230p locus
PbEif1a5.Rev	CTGGTGCTTTGAGGGGT	5' integration for 230p locus
PbHsp70Term.For	AAAATAAAAGGAGGGATCTAGAGAG	3' integration for 230p locus
Pb230p3.Rev	AGTGACTTTCAGTGAAATCGC	3' integration for 230p locus
BCDHKO.5'	CTTTGGGAATAGGCACCGATTTATAAATACTAG	BCDH undisrupted locus
BCDHKO.3'	CCTGGAGATGCCTTAACATTTCTTGG	BCDH undisrupted locus
KDHKO5'	CGCCTAAATGTAATTTTTCACGATAAATATTATGC	KDH undisrupted locus
KDHKO3'	CTCATTTATAACACCTTCAGTAATTGAATCACCC	KDH undisrupted locus

Table 3.1: DNA oligos used to generate constructs for lipoamidase and knockout experiments.

HprotKO.5'	GATTAAGAACTCTTAGAAAACAAGTATCCTGTAAGCG	H protein undisrupted locus
HprotKO.3'	CTTTTTCTTCTTTTGCTTCTTCTTCTTCGCAC	H protein undisrupted locus
BCDHKO.Int.5'	CTGACCCGAGTTCTATTTGAATATCC	5' integration primer for disruption of BCDH
BCDHKO.Int.3'	CCAATAATAATATCCTTGTTAGGGATGGAGTGC	3' integration primer for disruption of BCDH
KDHKO.Int.5'	GGGCACACATGCCCAGAG	5' integration primer for disruption of KDH
KDHKO.Int.3'	GGCGGAAAAGGAAGAAGAAGAC	3' integration primer for disruption of KDH
Hprot.Int.5'	CCTTTAATGCATACTACCATTAATAAATGG	5' integration primer for disruption of H protein
Hprot.Int.3'	CCTCTTAGTACTTTTTTGTCTTTTTGCGAACTC	3' integration primer for disruption of H protein
hDHFR.5'	GACGATGCAGTTTAGCGAACC	Cognate integration primer for 5' integration
hDHFR.3'	GGTGTTCTCTCTGATGTCCAGGAG	Cognate integration primer for 3' integration
GW2a	TGGGTGACTTTGGTGACAGATACTACTG	Integration primer for Plasmogem BCDH, and mE3 knockouts
PbEF1aREV	GTGAGCATTTAAAGCACAATATCTAGG	Integration primer for Plasmogem KDH and H protein knockouts
011169GT	ACATCGATTTTCGTCGCGCA	Integration primer for BCDH knockout with Plasmogem construct
011169QCR1	AGGCATCTCCAGGCACGAAA	Undisrupted BCDH locus
011169QCR2	GCAAACGTTCCACCAATAGCCCCA	Undisrupted BCDH locus
011169GT	AGACAAGAATGTTTTGAGGGAAGCT	Integration primer for KDH knockout with Plasmogem construct

Table 3.2: DNA oligos used to generate constructs for lipoamidase and knockout experiments.

110413QCR1	TGGATGGAAGAGAAGCCGTACA	Undisrupted KDH locus
110413QCR2	TTGCAATTTATGGGATGCCA	Undisrupted KDH locus
534891Gta	GGGTGGCTGGGCAAAA	Integration primer for H protein knockout with Plasmogem construct
534891QCR1	CCATCCATTGGTTTCAGAGCTTCCA	Undisrupted H protein locus
534891QCR2a	AGGAATTTTCAAAAGGGGTACAAGAAA	Undisrupted H protein locus
256413Gta	CTTACCACTTTGATGGCACCCTTT	Integration primer for mE3 knockout with Plasmogem construct
256413QCR1	TGCCCGCAACGAATACTGCA	Undisrupted E3 locus
256413QCR2a	CTAAGGAATCCACATGTTGTATGCAA	Undisrupted E3 locus
Hsp70.For	TGCAGCAGATAATCAAACTC	qPCR forward primer to measure total Pb population
Hsp70.Rev	ACTTCAATTTGTGGAACACC	qPCR forward primer to measure total Pb population
PShDHFR.For	GTGATTATGGGTAAGAAGAC	qPCR forward primer to measure transfected population
PshDHFR.Rev	TAAGGCATCATCTAGACTTC	qPCR forward primer to measure transfected population

Table 3.3: DNA oligos used to generate constructs for lipoamidase and knockout experiments.

Construct	Function	Successes	Attempts
pL1694-mLpaS236A	Attenuated mitochondrially targeted lipoamidase	1	1
pL1694-mKSA	Inactive mitochondrially targeted lipoamidase	1	1
pDEF-KDHKO	KDH E2 knockout	0	3
pDEF-BCDHKO	BCDH E2 Knockout	0	3
pDEF-HKO	H protein knockout	0	3
PbGEM-110413	PlasmoGem KDH E2 knockout	1	1
PbGEM-011169	PlasmoGem BCDH E2 knockout	1	3
PbGEM-011169mChFusion	hDHFR/mCherry/yFCU modified cassette of PbGEM-011169	0	2
PbGEM-011169mChSTOP	hDHFR/mCherry modified cassette of PbGEM-011169	0	2
PbGEM-534891	PlasmoGem H protein knockout	0	1
PbGEM-256413	PlasmoGem mitochondrial E3 knockout	1	1

Table 3.4: Summary of *P. berghei* transfections

	Hsp70	hDHFR
1 ng gDNA	30.58	29.02
10ng gDNA	26.75	23.19
NTC	N/A	26.94

Table 3.5: Mean values of qPCR performed in triplicate on BCDH KO gDNA. Ct values for 10 ng of gDNA are likely the most valid in the set, suggesting approximately 1/8th the parasite population bears the knocked out locus. However, repeated attempts at the experiment resulted in significant Ct values appearing in the nontemplate control (NTC), giving values around 26. This would suggest that the Ct value for the hDHFR loaded with gDNA is likely artificial, and that the true proportion of mutants is below 1/8th the total population. transfections

3.9 Figures

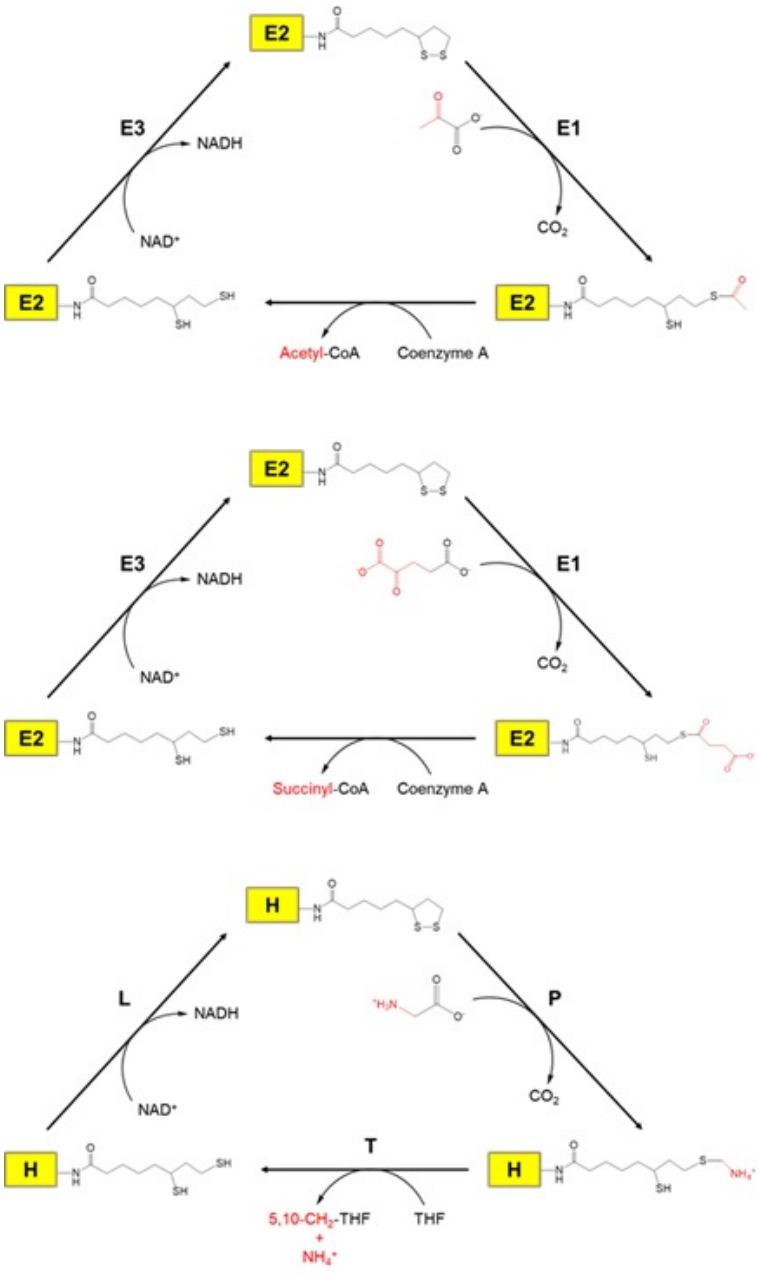

Figure 3.1: Canonical lipoate-mediated metabolism in a pyruvate dehydrogenase, alpha-ketoglutarate dehydrogenase and the glycine cleavage system. Decarboxylation and ligation of the substrate is performed by the E1 subunit (or the P protein). The E2 subunit (or the H and T proteins) attach the remaining substrate onto a cofactor. Finally, the E3 (or L protein) consumes NAD^+ in order to regenerate the oxidized lipoyl moiety on the E2 subunit (or H protein).

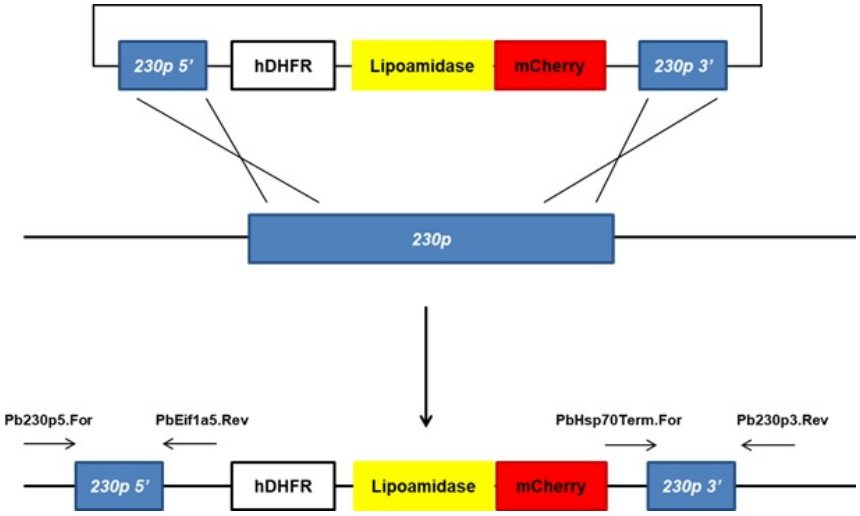

Figure 3.2: Knockin construct for lipoamidase into the dispensable 230p locus in *P. berghei*. Decarboxylation and ligation of the substrate is performed by the E1 subunit (or the P protein). The E2 subunit (or the H and T proteins) attach the remaining substrate onto a cofactor. Finally, the E3 (or L protein) consumes NAD^+ in order to regenerate the oxidized lipoyl moiety on the E2 subunit (or H protein).

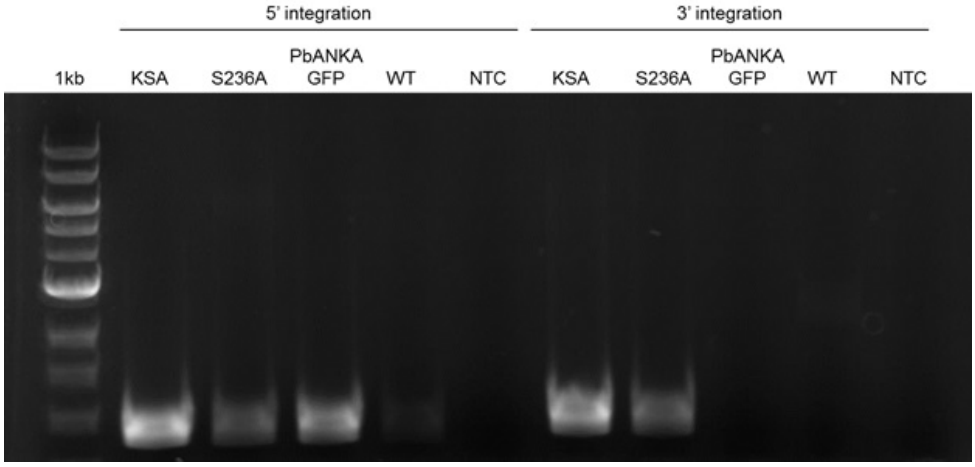

Figure 3.3: Both the KSA and S236A lipoamidase variants can be knocked into the 230p locus. PCR products of the appropriate size were produced for the KSA and S236A using genomic DNA from the mixed parasite population. *Pb*ANKA-GFP gDNA was used as a positive control for 5âĂŹ integration since this line was created using a similar strategy to insert GFP into the 230p locus. *Pb*ANKA gDNA was used at the WT lanes as a negative control.

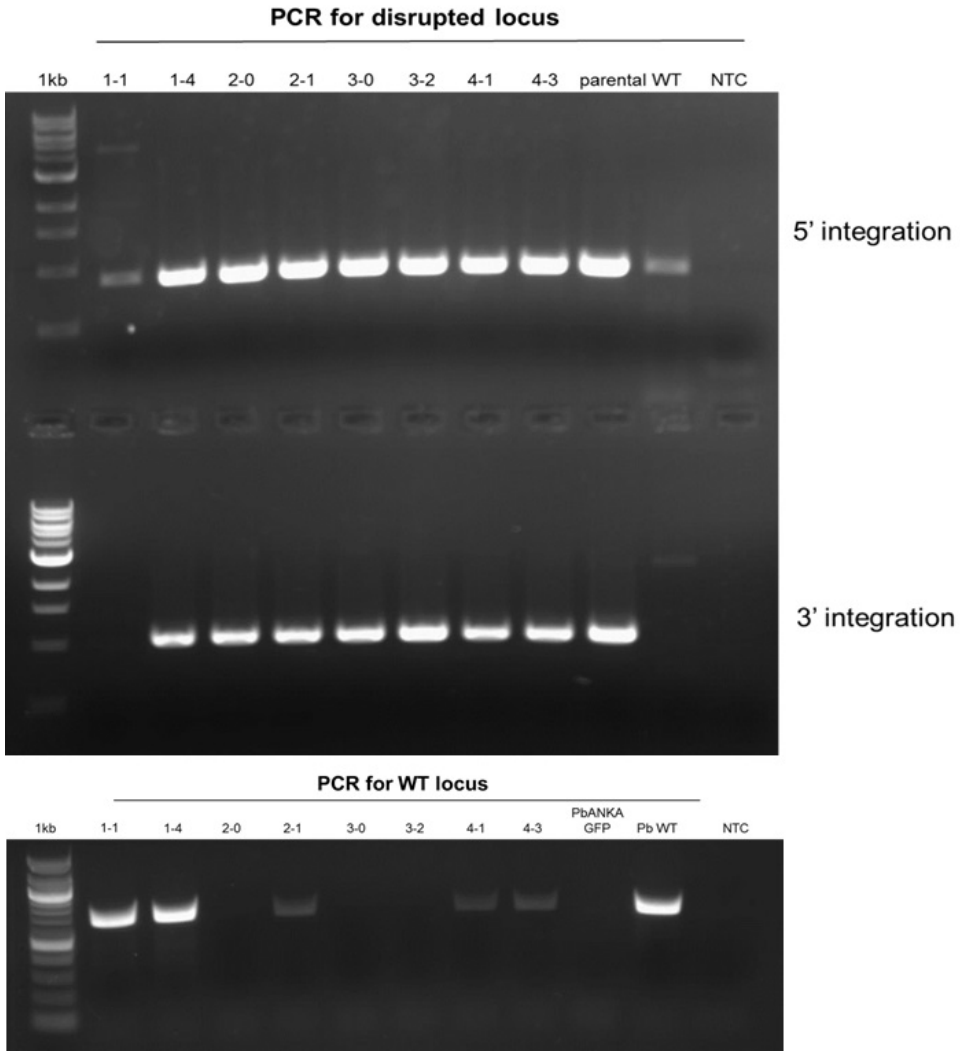

Figure 3.4: The *P. berghei* line expressing the inactive lipoamidase variant (KSA) was successfully single cloned. 8 out of 20 mice became patently infected during single cloning, and 7 came up positive for the integrated locus, and 3 were devoid of the wild type locus. This implies that some mice ended up getting more than one parasite each during the experiment. The parental line was used as a positive control for integration PCR, while both *Pb*ANKA-GFP and *Pb*ANKA were used as negative controls for PCR for the wild type locus.

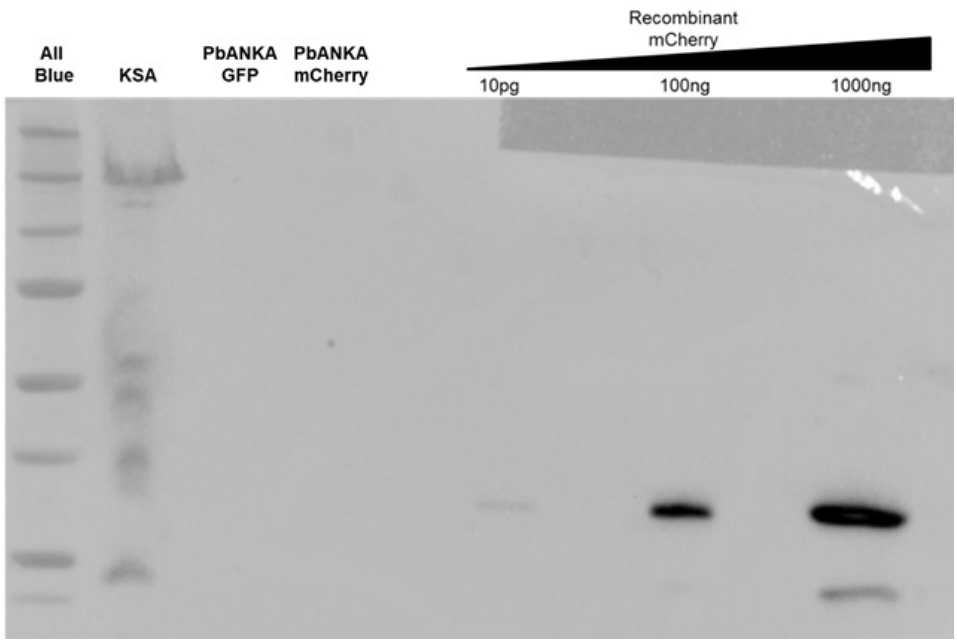

Figure 3.5: Inactive lipoamidase variant (KSA) mCherry fusion protein is well expressed.. The amino acid sequence suggests that the fusion protein should be seen at 110kD, but the predominant band appears at the 150kD mark. This is in line with previous observations that lipoamidase typically runs 30-40kD larger than predicted. *Pb*ANKA GFP lysate serves as a negative control, and *Pb*ANKA mCherry lysate and recombinant mCherry serve as positive controls. It is unclear why the *Pb*ANKA mCherry lysate used as a control does not show any bands.

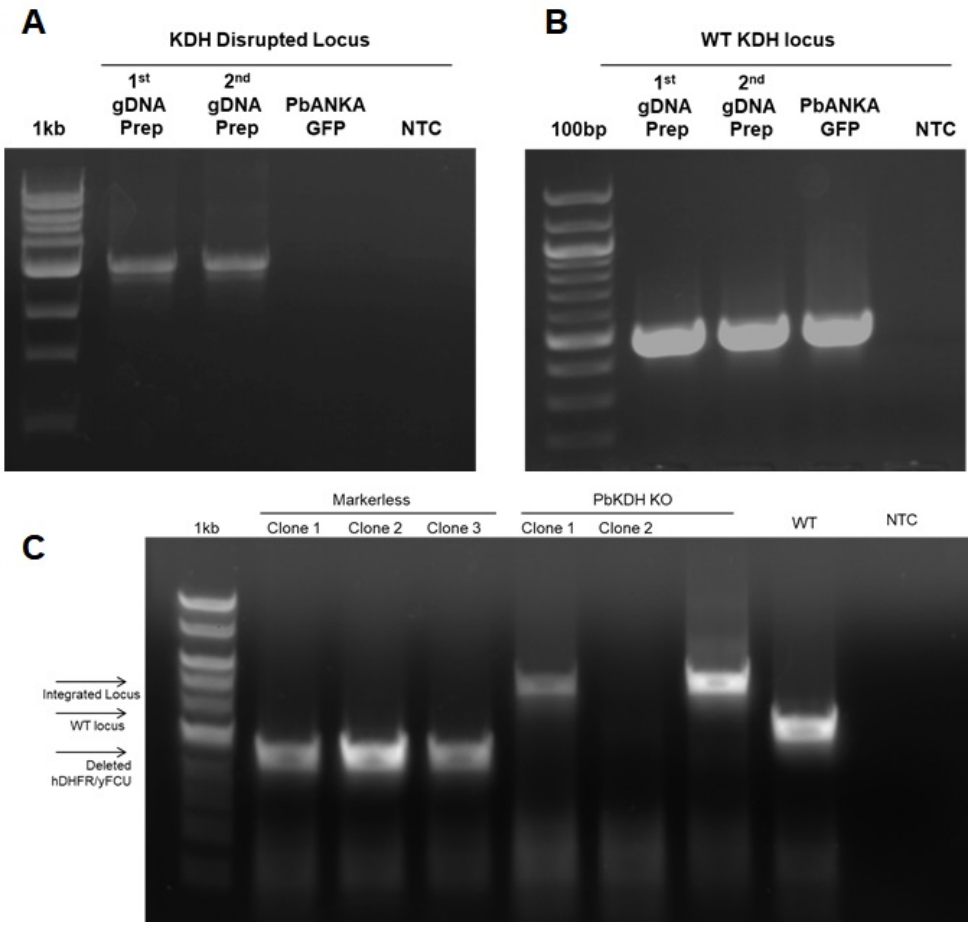

Figure 3.6: The KDH locus is disruptible in *P. berghei*. PCR demonstrates both the disrupted locus **A** and the WT locus **B**, suggesting that the parasite population is mixed, and that the KDH E2 locus is disruptible. Two gDNA preparations were tested since the first gDNA preparation was of poor yield. **C.**The PbKDH E2 KO line was successfully single cloned, and the markerless line was also generated and single cloned. Size shift PCR was used to investigate the KDH E2 locus and produce three possible bands: the wild type locus, integrated (knocked out) locus, and the integrated locus that excised the hDHFR/yFCU resistance cassette. Three clones that do not have either the KDH E2 locus or the hDHFR/yFCU cassette were generated. The next two lanes mark the two single clones generated from the initial single cloning experiment to isolate clones of the KDH E2 deletion. *Pb*ANKA GFP gDNA was used as a positive control for the wild type locus.

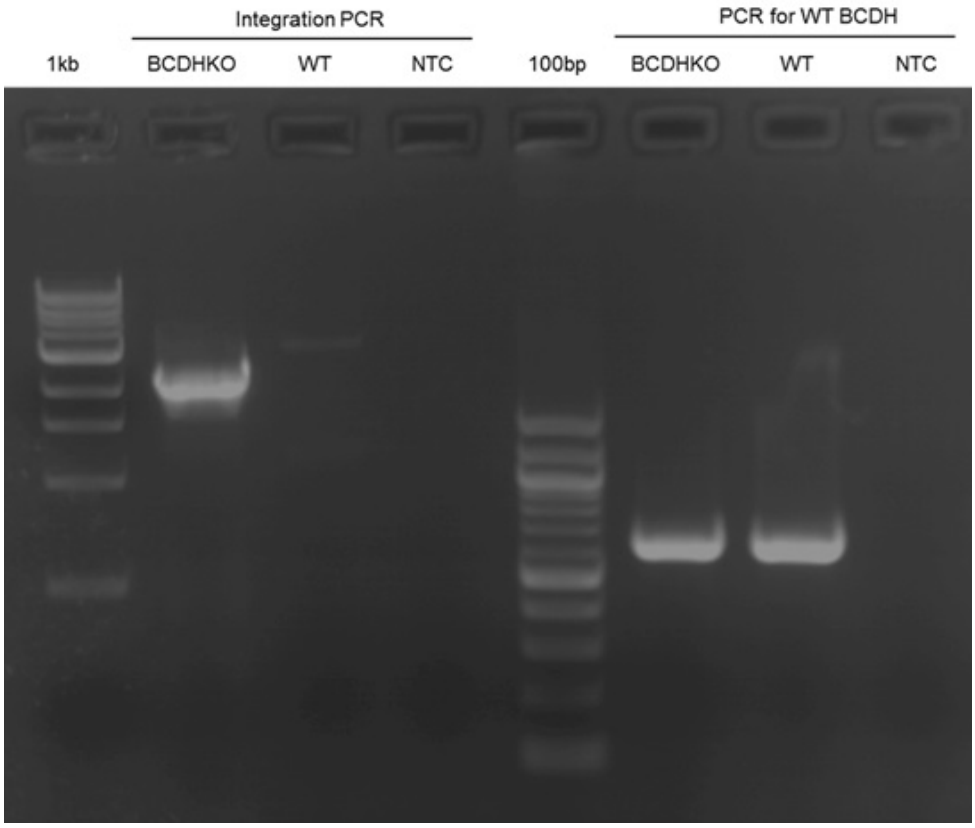

Figure 3.7: The BCDH locus is disruptible. PCR for the disrupted, integration locus is positive for the transfected population (left side of the gel), but is also positive for the wild type population (right side of the gel), suggesting a mixed population of parasites. The parental *Pb*ANKA-GFP gDNA was used as a negative and positive control, respectively

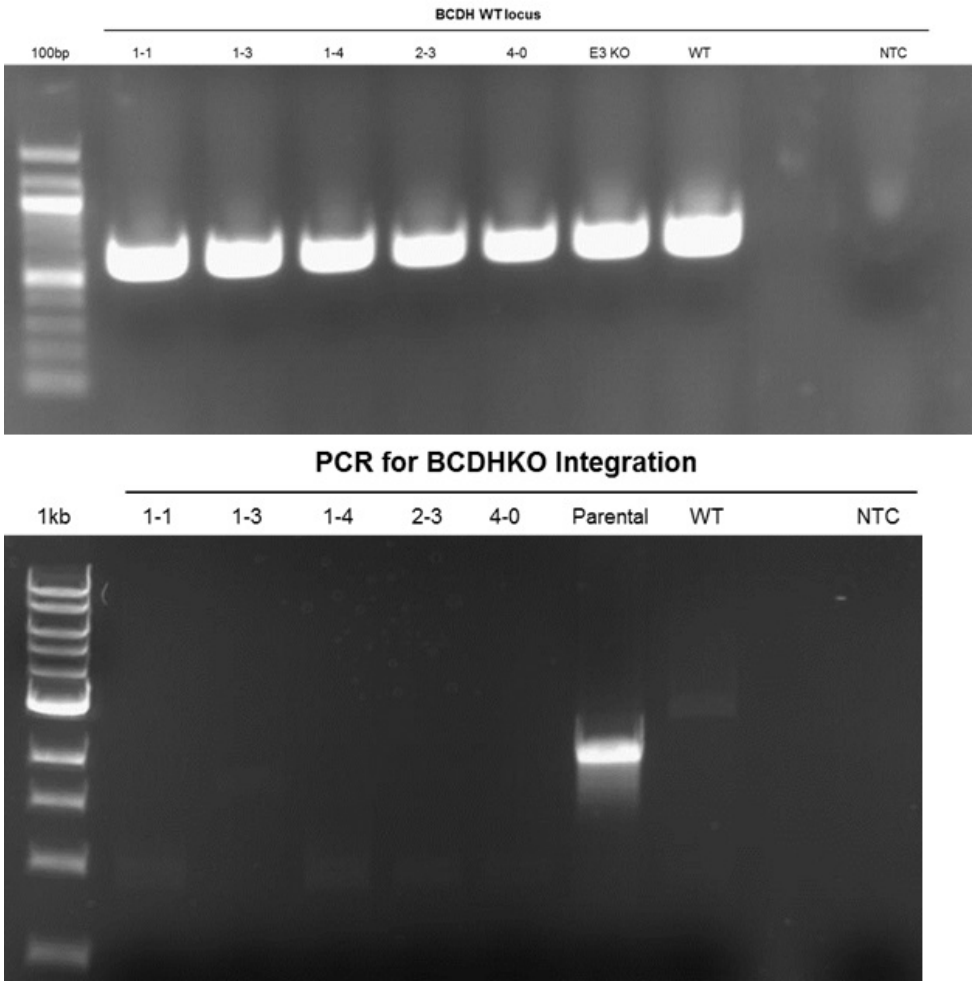

Figure 3.8: The BCDH knockout could not be single cloned.. PCR for the WT locus was performed (top gel) on parasites that came from five mice that became patently infected, showing positive signal for the wild type locus. gDNA from the E3 KO and the parental *Pb*ANKA-GFP lines were used as positive controls. PCR for the disrupted locus (bottom gel) was negative for all five parasite populations. The parental, mixed population gDNA was used as a positive control, and *Pb*ANKA-GFP was used as a negative control. This is a representative set of gels for three attempts at single cloning.

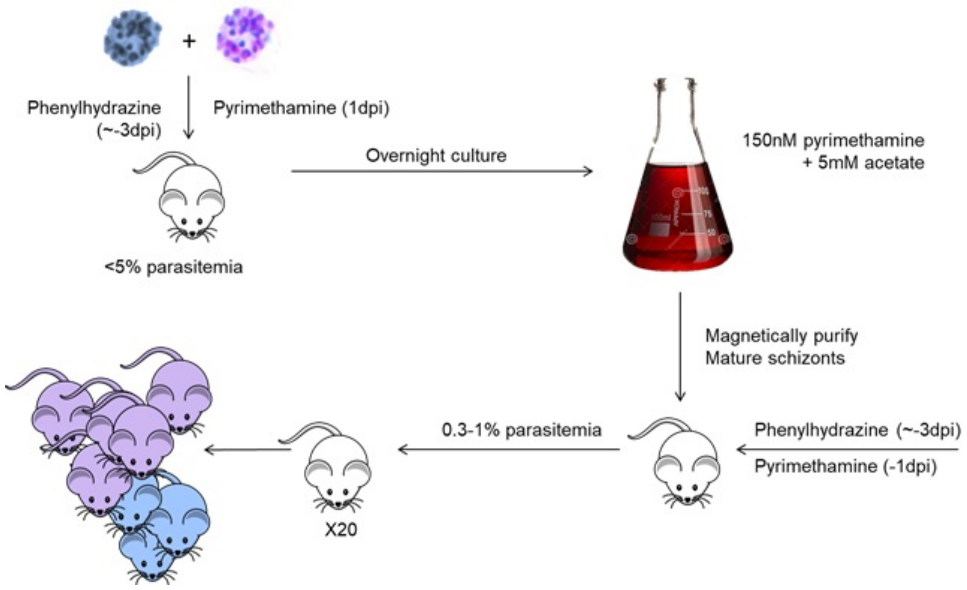

Figure 3.9: ***Ex vivo*** **culture attempt to enrich for transfectants.**.Continuous phenylhydrazine treatment was employed in order to enrich for reticulocytes, which are the preferred host erythrocyte for *P. berghei*, and possibly more so for this knockout. *Ex vivo* culture was supplemented with 150 nM pyrimethamine (approximately 10X the IC50 in culture) to suppress wild type parasites, and acetate was supplied to bypass potential toxicity of the knockout. Magnetic purification of schizonts was then performed to exclude wild type parasites, and continuous passage in pyrimethamine/phenylhydrazine treated mice was conducted to continue to enrich for the mutant parasites.

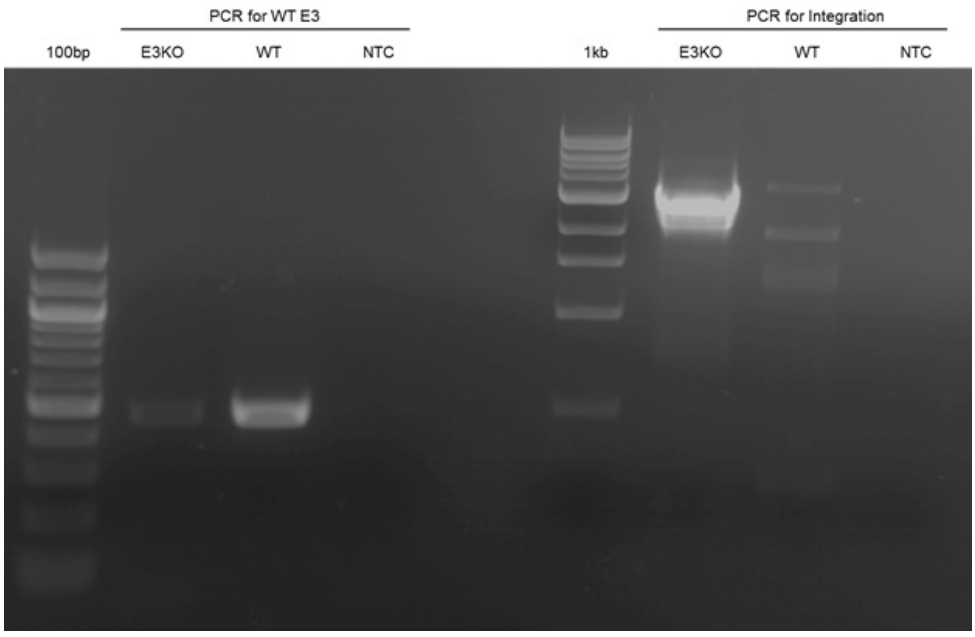

Figure 3.10: The shared, mitochondrial E3 subunit may be dispensible in the blood stage of *P. berghei*..PCR shows positive signal for both WT locus (left side of the gel) and the disrupted, integration locus (right side of the gel). *Pb*ANKA-GFP gDNA was used as a positive and negative control, respectively.

Chapter 4

The KDH, but not the BCDH, is disruptable in *P.falciparum*

4.1 Abstract

The lipoamidase experiments in *P. falciparum*, the results of the *P. berghei* knockout attempts, and other experiments demonstrated that mitochondrial lipoylation is essential for the blood stage of the malaria parasite. However, these experiments have not yet demonstrated which, or what combination, of the lipoylated complexes are essential in *P. falciparum*. Knockout experiments were performed in *P. falciparum* targeting the BCDH and the KDH to mixed results. We were able to disrupt the KDH, demonstrating that this protein is not essential for blood stage parasites, but we failed to delete the BCDH.

4.2 Introduction

Plasmodium falciparum has three, putative metabolic pathways in the mitochondrion that are lipoate dependent: the branched chain alpha-ketoacid

dehydrogenase (BCDH), the alpha-ketoglutarate dehydrogenase (KDH) and the glycine cleavage system. Work in our lab demonstrated that the parasite scavenges exogenous lipoate and attaches it to these enzyme systems (Allary et al., 2007). Abrogating these lipoylation pathways by knockdown (Wang et al., 2017), drug treatment (Afanador et al., 2014) or the lipoamidase experiments described in Chapter 2 demonstrate that lipoylation is essential for parasite growth, but it is unclear which or what combination of these enzymes is essential.

Metabolomic experiments demonstrate that both *P. falciparum* (MacRae et al., 2013) and *P. berghei* (Oppenheim et al., 2014) have a typical glycolytic pathway that is bridged to the mitochondrial citric acid cycle, also known as the tricarboxylic acid cycle (TCA), which fuels the electron transport chain (ETC). The parasite's near-exclusive use of glucose as a carbon-based energy source and the essentiality of the ETC (Painter et al., 2010) strongly suggest that a pyruvate dehydrogenase (PDH) exists to connect glycolysis to the TCA. The genomes of malaria parasites appear to encode two independent PDH enzyme complexes - one trafficked to the apicoplast and the other trafficked to the mitochondrion (BCDH). The ability to knockout the apicoplast lipoylation machinery, which should abrogate any apicoplast PDH activity, demonstrates that lipoate-dependent PDH catalysis is not essential to the parasite in the blood stage (Falkard et al., 2013; Günther et al., 2007). This conclusion is supported by the fact that the apicoplast resident PDH being dispensible in the blood stage of several species of *Plasmodium* (Pei et al., 2010). Consistent with these findings, metabolomic experiments demonstrate that the apicoplast

PDH is not the primary pathway for acetyl-CoA generation (Cobbold et al., 2013). The ability of acetate to rescue lipoamidase toxicity described in Chapter 2, the knockout experiments described in Chapter 3, and the E1 alpha knockout of the BCDH (Oppenheim et al., 2014) strongly implicate the BCDH as the chief source of acetyl-CoA for the parasite (and thus a mitochondrial PDH). These results imply that the lipoylated BCDH E2 subunit should be essential for blood stage parasite survival.

Available evidence suggests that all of the components of the KDH complex should be dispensable in blood stage malaria parasites. The E1 subunit of KDH has been deleted in *P. falciparum* and metabolomics analysis of this mutant shows that this step of the TCA pathway is not required for the survival of blood stage parasites (Ke et al., 2015). It is not clear, however, whether the lipoylated E2 subunit is required for some other function. Indeed, some bacteria use lipoylated enzyme complexes such as the PDH or KDH in oxidative stress pathways in addition to their canonical roles (Spalding and Prigge, 2010). In order to test whether or not these lipoylated proteins are essential in *P. falciparum*, we performed knockout experiments targeting the BCDH E2 and the KDH E2 proteins.

4.3 Results

4.3.1 The *Plasmodium falciparum* kdh locus is disruptable

Cloning and implementing a double crossover strategy at the time this work was done would have required much more time and effort than a single crossover strategy, so using single crossover was chosen as a pilot

experiment to see whether the *kdh* gene is disruptable. The homology arm used separates the lipoylation domain from the catalytic domain of the KDH with the expectation that if the disrupted locus still produced mRNA that could be successfully translated, it would produce a fragmented protein that could be lipoylated, but unable to perform catalysis (**Figure 4.1**). The single crossover was designed in such a way that an HA tag would be appended to the 3' end of any truncated protein product. This single KDH homology arm was cloned into the pCC1s single-crossover plasmid for transfection of *P. falciparum* 3D7-GL strain parasites. This line was chosen because it is capable of producing functional gametocytes, allowing us to study mosquito stage effects if blood-stage knockouts were viable.

After several weeks of drug-cycling with anti-folate drug WR99210 (**Figure 4.2**), diagnostic PCR products demonstrated that the kdh locus was successfully disrupted (**Figure 4.3A,B**). Multiple attempts at single cloning a parasite that harbored only the disrupted locus from the mixed population failed, resulting in 36 parasite clones with complex genotypes. After expansion from a single parasite cell, these lines contain both the wild type and disrupted locus (**Figure 4.4**). Additionally, there is PCR evidence for episomally maintained plasmid in these lines, indicative of spontaneous plasmid excision. This phenomenon persisted after multiple attempts at drug cycling which should have favored the growth of parasites with integrated plasmid rather than episomally maintained plasmid. Presumably, KDH disrupted parasites excise the transfection plasmid at some rate (through crossover of the homology arm with its merodiploid copy), allowing us to detect the wild type locus and the

plasmid over time. Taken together, these results suggests that the *kdh* gene can be disrupted, and that the KDH E2 protein is not essential for blood stage parasite growth; however, we also conclude that we will not be able to obtain clonal parasite populations based on facile plasmid excision.

4.3.2 The *bcdh* locus is not disruptable

A 645 nucleotide section of the coding region of the *bcdh* gene was amplified using the same design philosophy as the KDH into the pCC1s single crossover plasmid. Multiple transfection attempts yielded no viable parasites at up to two months into negative selection. Transfection into alternative parasite lines (3D7 and NF54) and with acetate supplementation came to little success, with two successful transfections coming up, but without PCR evidence for the disrupted locus (**Figure 4.6A, B**). These parasite lines also did not display evidence of episomal maintenance (**Figure 4.7**), suggesting spurious integration into a different part of the parasite genome. Since the transfection plasmid contains 5' and 3' UTR elements cloned from the *P. falciparum* genome, these are likely regions for spurious integration (López-Estraño et al., 2007). Based on multiple failures to target the bcdh locus in multiple parasite strains both with and without acetate supplementation (**Table 4.2**) suggests that BCDH E2 protein is essential for blood stage parasite growth.

4.4 Discussion

Although the parasite does appear to tolerate disruption of the kdh gene, the inability of the parasite to stably maintain the knockout plasmid integrated

into the genome may suggest plasmid excision is occurring, a phenomenon previously described in the literature (Jacobs-Lorena et al., 2010; Knuepfer et al., 2017; Sultan et al., 1997), due to the use of the single-crossover approach. Because the disruption can occur, but the plasmid appears to be regularly excised, there may be some slight fitness defect associated with loss of KDH activity. It should be noted, however, that though multiple and variant methods to produce single clones were used, it is still a formal possibility that the clones obtained were simply not single clones.

This does not necessarily contradict the findings of the *Pfkdh* E1 knockout (Ke et al., 2015). Analysis of TCA cycle metabolites in the various knockout lines made by Ke and coworkers showed that nearly every knockout was accompanied by biochemical compensation feeding into and out of the citric acid cycle as a result of TCA gene disruption. Although this compensation may allow the parasites to survive in the absence of a TCA cycle enzyme, the altered metabolic states may come with some small fitness costs. In the case of ablating KDH activity, the route of glutamate/glutamine carbon into the TCA cycle (as alpha-ketoglutarate) is no longer functional. Considering the importance of glutamine and glutamine as the major carbon input for the citric acid cycle (MacRae et al., 2013; Srivastava et al., 2016), it is reasonable to predict that the loss of KDH activity will affect parasite fitness.

The failure to knockout the BCDH in multiple strains of *P. falciparum* suggests that the gene is important, and possibly essential, for the parasite. Based on the hypothesis that BCDH generates acetyl-CoA, this would imply that acetyl-CoA generated in the mitochondrion is essential for mitochondrial

and/or cytosolic metabolism. Since the only known role of acetyl-CoA in the mitochondrion is to act as a carbon source for the dispensable TCA cycle, it seems likely that acetyl-CoA generated by BCDH is important for cytsosolic metabolism. We attempted to supplement cytosolic acetyl-CoA production by providing 5 mM acetate in the parasite medium with the hope that the cytosolic acetyl-CoA synthetase would supply the required metabolite and allow us to disrupt the bcdh gene. The inability of acetate supplementation to bypass loss of BCDH function suggests that cytosolically generated acetyl-CoA is insufficient in compensating for loss of mitochondrially generated acetyl-CoA. In addition, although the KDH may moonlight as a PDH, (Chan et al., 2013; Foth et al., 2004), any potential ability for the KDH to compensate loss of the BCDH may also be insufficient to rescue the effects of the BCDH knockout.

Comparisons to the experiments done in *P. berghei* in the previous chapter may be informative. The successful single cloning of a stable knockout of the kdh E2 gene was perhaps possible because the technique of double crossover homologous recombination with a linearized plasmid was used, eliminating the possibility of plasmid excision. Since this method permanently deletes part of the targeted gene, there is no way for the parasite to recover the function of the knocked out protein, even if there is some fitness defect associated with its loss. The ability to disrupt the *Pbbcdh E2* locus, but not recover a single clone would suggest that knockout in *P. falciparum* may be similarly difficult. The reticulocyte preference characterized in the *P. berghei* BCDH E1 knockout (Oppenheim et al., 2014) that may be shared with the E2 knockout

would suggest that the parasite would prefer the more metabolically rich environment of a reticulocyte (Srivastava et al., 2015) in order to compensate for loss of BCDH activity. Tissue culture work with blood stage *P. falciparum* does not typically include reticulocytes, making it more difficult for the parasite to tolerate disruption of BCDH activity, even with acetate supplementation.

Although useful comparisons can be made between human and murine parasite species, some fundamental metabolic pathways differ in significance. For example, phenotypes differ between knockouts of fatty acid synthesis genes in different parasite species (van Schaijk et al., 2014; Yu et al., 2008) and the same is true for coenzyme A synthesis genes (Hart et al., 2016; Saliba et al., 1998). Similarly, knockout of aconitase, another enzyme of the citric acid cycle two steps upstream of the KDH, was not described as having a growth phenotype in *P. falciparum* (Ke et al., 2015), but has a severe growth phenotype in *P. berghei* (Srivastava et al., 2016). In addition, the ability for *P. berghei* to invade metabolite enriched reticulocytes (Srivastava et al., 2015) may mask potential auxotrophies that are more evident in *P. falciparum* in tissue culture. Because of these complications, knockout experiments must be comprehensively performed within a species to fully understand their respective metabolic requiements.

In addition to spontaneous plasmid excision, parasites appear to be able hold onto a plasmid at least up to a year after removing drug pressure (O'Donnell et al., 2001), making drug cycling a possibly fruitless endeavor. Long term culturing of episomal populations is also problematic, as plasmids have regulatory elements that may be homologous to dispensable genomic

regulatory elements, allowing parasites to integrate spuriously at those loci (López-Estraño et al., 2007). Finally, parasites appear to be able to traffic plasmids to non-plasmid-bearing parasites via exosome trafficking (Regev-Rudzki et al., 2013). Both individually and in combination, these confounders contribute to unwanted uncertainty when attempting to use single-crossover, especially when targeting genes that may be important for the parasite. This suggests that a more targeted and robust system, like the recently successful Cas9-mediated genome editing technique (Ghorbal et al., 2014; Wagner et al., 2014) would be more successful in generating knockouts and clones of KDH E2 and possibly the BCDH E2 in conjunction with acetate supplementation. This genome editing technique forces a targeted double-strand break and highly increases the frequency of a double-crossover homologous recombination event that cannot later be reversed.

No attempt was made to disrupt the H protein in *P. falciparum*, due to the small size of the protein's coding region, and thus, the inability to try a single crossover deletion. Considering the results observed in the previous chapter in attempting the parallel knockout in *P. berghei*, and the apparent essentiality of *Pb*LipL1 (Wang et al., 2017), the enzyme responsible for lipoylating the H protein, it would seem that the H protein locus may be refractory to knockout in *P. falciparum* as well. As discussed in the previous chapters, why this particular protein is essential is a matter for speculation.

4.5 Future Directions

4.5.1 Interrogating canonical lipoate-dependent biochemistry in *Plasmodium falciparum*

The hypothesis guiding this project was that lipoylation of at least one of the mitochondrial substrates was essential for parasite growth. In light of the experiments performed here, the rest of the thesis, and the current literature, in order to turn this project into a finished publication, knockout experiments should be performed for all members of lipoate-dependent biochemistry in the mitochondrion: the substrates (BCDH E2, KDH E2 and the H protein), the lipoylation machinery (LipL1 and LipL2) and the recycling subunit (the E3 subunit). Knockouts should be feasible for the BCDH, the KDH, LipL2 and the mitochondrial E3 subunit. Knockouts should be feasible for the BCDH, the KDH, LipL2 and the mitochondrial E3 subunit using Cas9 mediated knockout because of the advantages discussed above. Parasite work should be continued in a gametocyte competent line as performed above (3D7, 3D7-GL, NF54)) in order to preserve the ability to interrogate mosquito stage phenotypes. Acetate supplementation should be used for these transfections, considering the acetate dependency observed in the lipoamidase experiments. It should also be exercised in the KDH knockout attempt as the KDH may participate in the conversion of pyruvate to acetyl-CoA (Chan et al., 2013).

These particular knockouts should be characterized for growth against parental lines, with and without acetate supplementation. Considering the difficulty of getting a KDH knockout clone, it is possible that there may be a small, but measurable growth phenotype that would provide *in vivo*

evidence that the KDH complex can moonlight as a PDH. Because of this possible phenomenon and the likelihood that the BCDH is the primary system performing pyruvate-to-acetyl-CoA catalysis, a double knockout should also be performed because a double knockout may be synthetically lethal, due to the possibility that both the BCDH and the KDH can convert pyruvate to acetyl-CoA.

Western blot and/or autogradiography should be used to confirm absence of lipoylation by knocking out these genes: knockout of the BCDH and KDH should result in disappearance of the lipoyation band for these respective proteins, while knockout of the LipL2 should result in disappearance of both the BCDH and KDH bands. This assay on the BCDH and KDH knockouts would provide definitive evidence that the lipoylated proteins identified in previous literature (Allary et al., 2007) are indeed the BCDH and KDH E2 subunits. Performing similar experiments with the LipL2 knockout would strengthen the model that LipL2 is the enzyme responsible for lipoylating the BCDH and KDH.

Biochemical work should be performed to confirm the biochemistry suggested in these knockout studies. Plasmid shuffle experiments in yeast may be the easiest way to demonstrate this, as the yeast knockout library is readily available, yeast media reagents are available in the lab, and data should be relatively easily generated; the experiments would only require cloning, transfection of yeast and overnight growth on combinatorially variable agar plates with various media supplements, such as acetate and branched chain amino

acids. Yeast rescue experiments using *P. falciparum* homologs to rescue homologous yeast knockouts coupled to dilution series experiments can provide a wealth of data for publication and strengthen the hypotheses regarding the activities of the BCDH and KDH and the lipoylation enzymes. Purification of the recombinant BCDH E1 subunit and performing kinetics and binding assays against branched chain alpha-ketoacids and pyruvate should also be performed to provide biochemical evidence that the BCDH can catalyze the PDH reaction in addition to, or instead of, the BCDH reaction.

All these experiments can be performed independently of the success of the knockouts, allowing for data generation without waiting for knockouts, which can take weeks to transfect, clone and validate. This basic metabolic work can be supplemented with mass-spectrometry-based metabolite analysis in parasites using labeled glucose and glutamine for all knockouts to confirm the affected biochemical pathways.

All the above experiments should fill in the gaps in our understanding of mitochondrial lipoate-dependent biochemistry with *in vitro* confirmation of the BCDH's biochemistry, and *in vivo* evidence to confirm the biochemistry regarding lipoylation and lipoate-dependent catalysis.

4.5.2 Interrogating non-canonical lipoate-dependent biochemistry

The experiments describe above should be sufficient to publish a paper that looks at canonical lipoate-mediated catalysis. The other genes associated with this project address what is most likely noncanonical biochemistry, and

will thusly be exploratory in nature.

It is likely that LipL1 and the H protein will be refractory to knockout considering the experiments attempted in this thesis in *P.berghei* and in the literature. Regarding genetic work, knockdown or a novel inducible knockout tool would be required to confirm that these genes are essential. Previous attempts to knockdown the LipL1 using tetR repression (Wang et al., 2017) resulted in nominal success, meaning that alternative knockdown tools need to be used, e.g. the auxin induced degron (Kreidenweiss et al., 2013), TetR DOZI (Ganesan et al., 2016), or the knock-sideways method (Birnbaum et al., 2017) to more fully determine if these genes are important for parasite survival. Western blot and autoradiography can be used here as well to observe the hypothesized reduction in lipoylation.

Fluorescence microscopy can be used to interrogate the hypothesis that lipoate-mediated biochemistry is important for redox homeostasis. A reporter line has been generated to use fluorescence to determine the glutathione redox potential in the parasite mitochondrion (Mohring et al., 2017) - this can be used as the parental line for any or all of the genetic modifications described above. This microscopy can be performed on live cells, adding the possibility of challenging the different parasites knockouts/knockdowns with various oxidative insults tosee how the loss of lipoate-related proteins affects the response. Live or immunofluorescence microscopy can be used with MitoTracker to see if these proteins are important for maintaining the mitochondrial membrane potential, as demonstrated with microscopy performed against parasites treated with atovaquone and proguanil (Painter et al.,

2007). Finally, mass spectrometry can be performed on the lipoylated proteins to see if non-canonical modifications are made on lipoate, e.g. oxidation or nitrosylation, to see if these molecules are used as redox sinks.

Ultimately, pulldown experiments need to be performed on the H protein in order to generate other hypotheses for the role of the H protein. This experiment would demonstrate how much of the H protein is lipoylated, what covalent modifications occur on the lipoate moiety, and under what circumstances these may change, e.g. redox insult. It would also be useful to find out what binding partners the H protein may have in order to generate further hypotheses.

4.6 Methods

4.6.1 Knockout Constructs

The disruption constructs for the *bcdh* and the *kdh* were made by PCR amplifying homologous regions from gDNA from the 3D7 line of parasites using primer pairs KDH.KO.F/KDH.KO.R and BCDH.KO.F/BCDH.KO.R, respectively (See **Table 4.1** for primer sequences). Homologous sections of about 600 base pairs were designed to begin immediately after the nucleotides coding for the lipoylated lysine and excluding essential regions at the end of the catalytic domain. The homology arms also contained an in-frame stop codon (**Table 1** red text) to block translation of the downstream merodiploid copy, even if it was spuriously transcribed. The PCR products were ligated into the pCC1s single crossover disruption plasmid using restriction sites *Bgl*II and *Xho*I, generating plasmids pCC1s-BCDHKO and pCC1s-KDHKO. PCR

primers were used to verify integration, plasmid retention and presence of the wild type locus.

4.6.2 Parasite Transfection and Selection

Red blood cells were preloaded with 75 μg of plasmid by electroporation and added to the 3D7-GL, 3D7 or NF54 line. After one or two days, parasites were selected by treatment with 5 nM WR99210. In successful transfections, parasites would appear between 4-6 weeks of selection, at which time they were PCR confirmed for episomal maintenance of the transfection plasmid. Parasites were cycled on and off WR99210 (2 weeks on, 2 week off) until parasite growth kinetics more closely mimicked that of the parental line (parasitemia increasing by a factor of 8 per two day growth cycle) (**Figure 4.2**). Once genomic integration was detected by PCR, parasites underwent single cloning through limiting dilution in 96 well plates, after which clones were characterized for by PCR to detect the disrupted locus, the wild type locus, and/or presence of the pCC1s plasmid.

4.7 References

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4.8 Tables

Name	Sequence	Purpose
BCDH.KO.F	GGTGGTAGATCTTAATTAAAAGTTGGATCATATTTCTGTGAGATAG	Generate the BCDH homology region
BCDH.KO.R	GGTGGTCTCGAGTCCATGAGGAGTATCAACAGCTATAG	Generate the BCDH homology region
PfBCDHKO.5'Int	GGAGAGTTTGTTGACTGTACAAAGTGAC	BCDH KO 5' integration primer
HACC1-727R	GCTATTTACATGCATGTGCATGCAC	Shared 5' integration primer
PfBCDHKO.3'INT	GCTCCAAAATTACTAACAGTTATTGTACC	BCDH KO 3' integration primer
pCC1d REV1	GATATTTTTATAATAAACCAATAGATAAAATTTGTAGAG	Shared 3' integration primer
KDH.KO.F	GGTGGTAGATCTTAAGTTAGTGTTGATATAAATTCTAAAGTAAGTGG	Generate the KDH homology region
KDH.KO.R	GGTGGTCTCGAGGCCATTTGGTGTAGCTACTGCTACAG	Generate the KDH homology region
PfKDHKO.5'Int	GCAAATATTGAAGGATCACTTAAGAGATATTTTTC	KDH KO 5' integration primer
PfKDHKO.3'INT	CTCCATTAGATATTGTAAATGTACCACC	KDH KO 3' integration primer

Table 4.1: Primers used for this project. Endonuclease sites are marked in blue text while translational stop codons are marked in red text.

Construct	Parasite Line	Transfection Success
pCC1S-KDHKO	3D7-GL	1/1
pCC1S-BCDHKO	3D7-GL	0/5
	3D7-GL + acetate	2/2
	3D7	0/1
	NF54	0/2

Table 4.2: Transfection summary. Transfection success is defined as parasites coming up before day 40 of transfection.

4.9 Figures

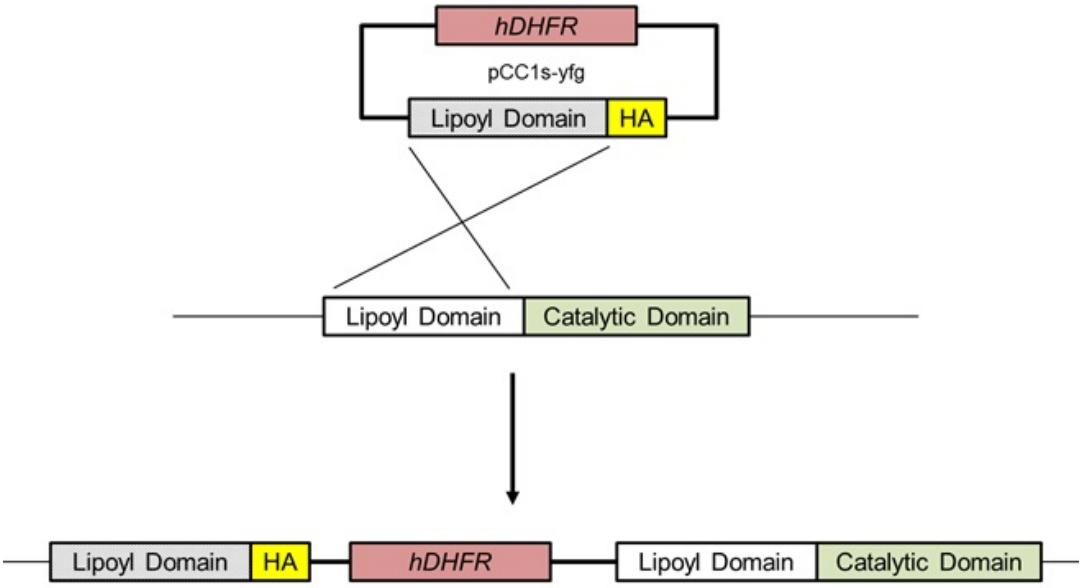

Figure 4.1: Schematic of single-crossover disruption strategy. Endonuclease sites are marked in blue text while translational stop codons are marked in red text.

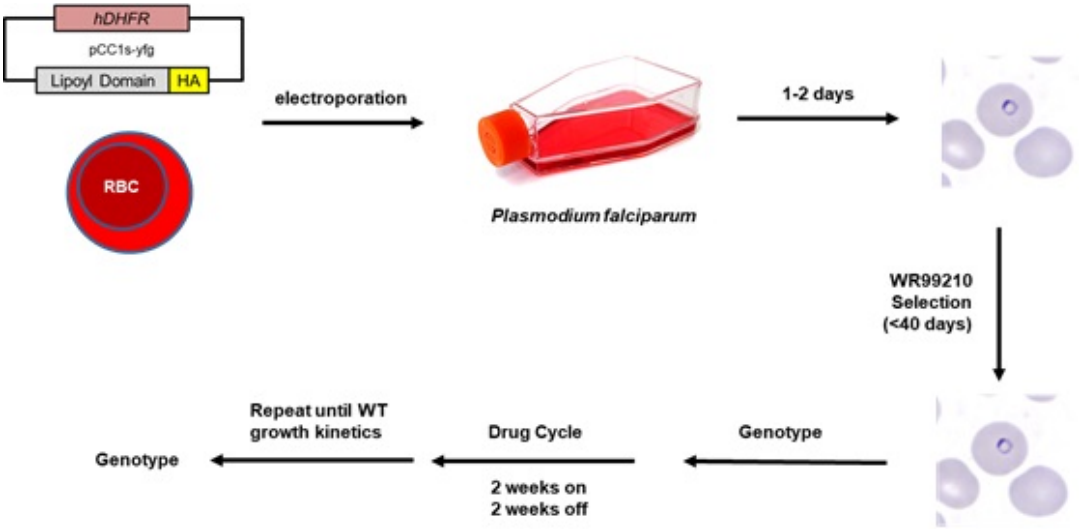

Figure 4.2: Workflow for gene disruption in *P. falciparum*. Parasites should be stably transfected with the single-crossover plasmid, and then selected with WR99210 for several weeks to produce resistant parasites. Parasites are then genotyped for episomal plasmid retention or disruption of the locus, and then drug cycled to eliminate parasites that lose the plasmid and enrich for parasites with plasmid integration.

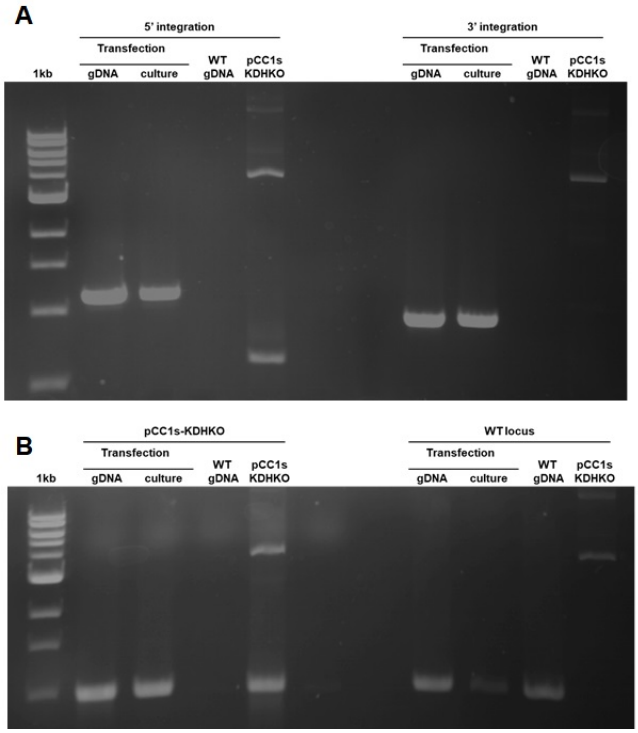

Figure 4.3: The *kdh* gene is disruptable. A. PCR for 5′ and 3′ integration of the plasmid into the KDH locus was performed on the mixed population of parasites, either as a gDNA preparation or from a 20 μl sample from culture. The gel demonstrates bands of the appropriate size, demonstrating integration of the entire pCC1s-KDHKO plasmid into the KDH locus. Genomic DNA of the parental WT line (3D7-GL) and the plasmid used for integration were used as negative controls. **B.** Parasite culture was of mixed genotype. PCR was performed to determine presence of the knockout plasmid and the WT KDH locus on the same templates used in 1A. The gel demonstrates bands indicating presence of the plasmid and the undisrupted WT locus, showing that the population is of mixed genotypes: WT parasites, parasites harboring the pCC1s-KDHKO plasmid and parasites with the integrated locus as demonstrated in the previous figure panel. This time, genomic DNA of the parental WT line and the pCC1s-KDHKO plasmids were used as positive controls for their respective PCR reactions.

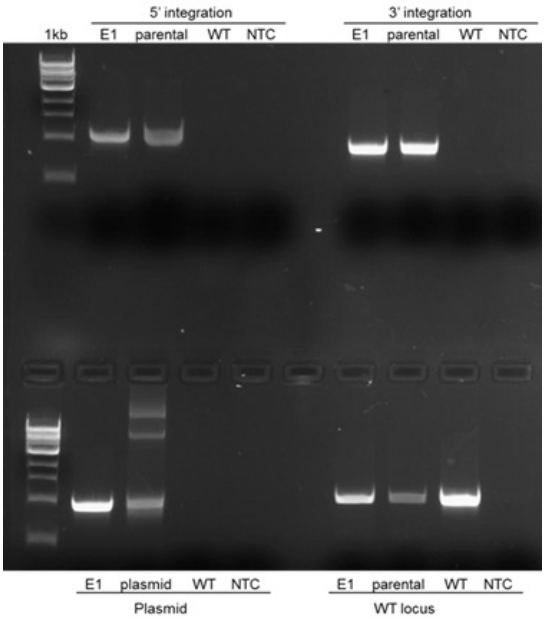

Figure 4.4: KDH knockout clone could not be isolated. Representative PCR of one of the KDH KO clones, clone E1. The top half of the gel shows that clone E1 is PCR positive for the integrated locus. The parental, mixed population was used as a positive control. The bottom half of the gel shows that parasites are PCR positive for both the plasmid (pCC1s-KDHKO) as well as the WT locus. This would suggest that either the clone retrieved was not a single clone, or that the plasmid can be efficiently excised and/or reintegrated.

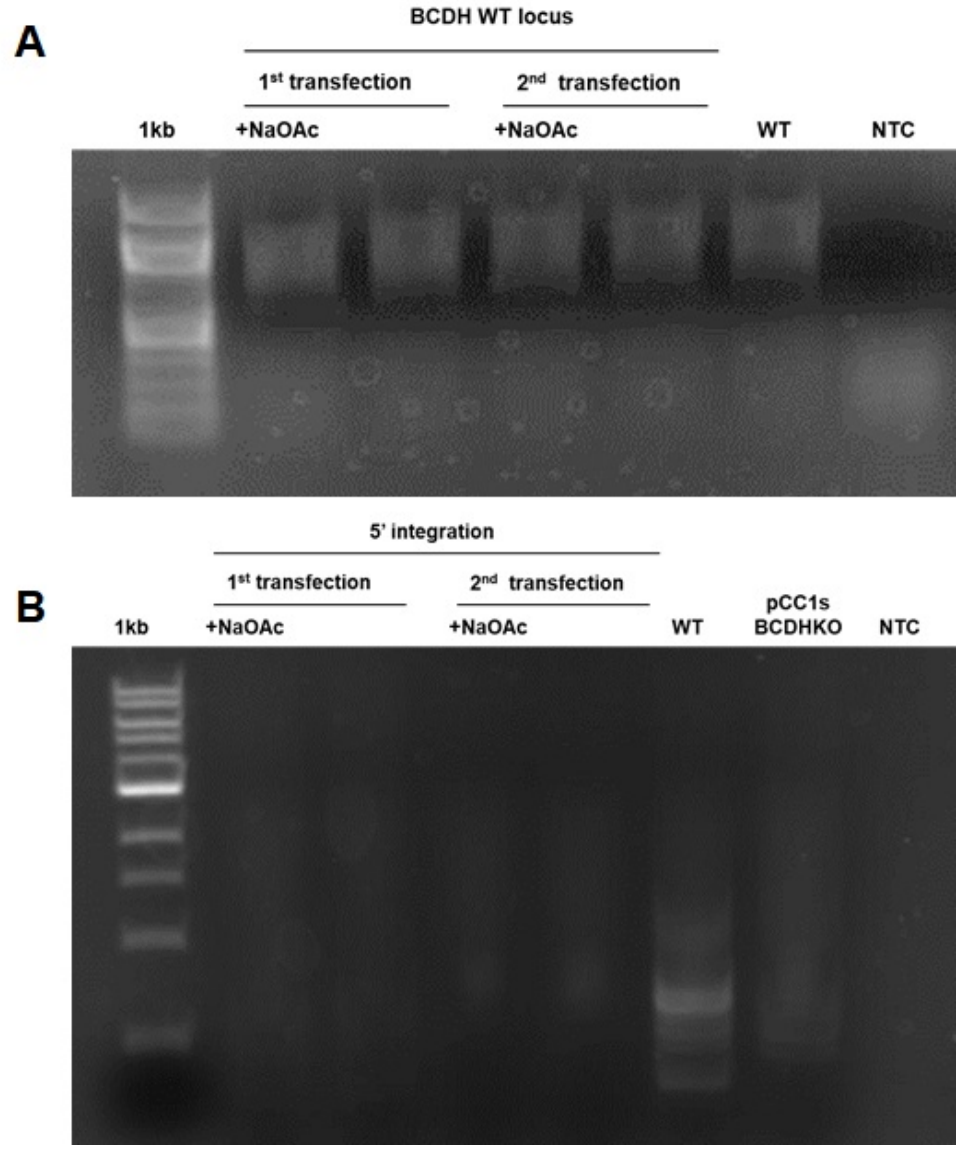

Figure 4.5: BCDH locus is refractory to targeting. A The BCDH locus was retained in two different transfections. 3D7-GL parasites were transfected with and without acetate supplementation, and drug cycled until parasite growth more closely resembled wild type kinetics. PCR to identify the BCDH locus demonstrates that the locus remains undisrupted in all 4 lines. Parental gDNA was used as a positive control. **B** There is no PCR evidence of integration of the plasmid into the BCDH locus in any of the four lines. Both WT gDNA and the transfection plasmid were used as negative controls; bands that appear are likely spurious products.

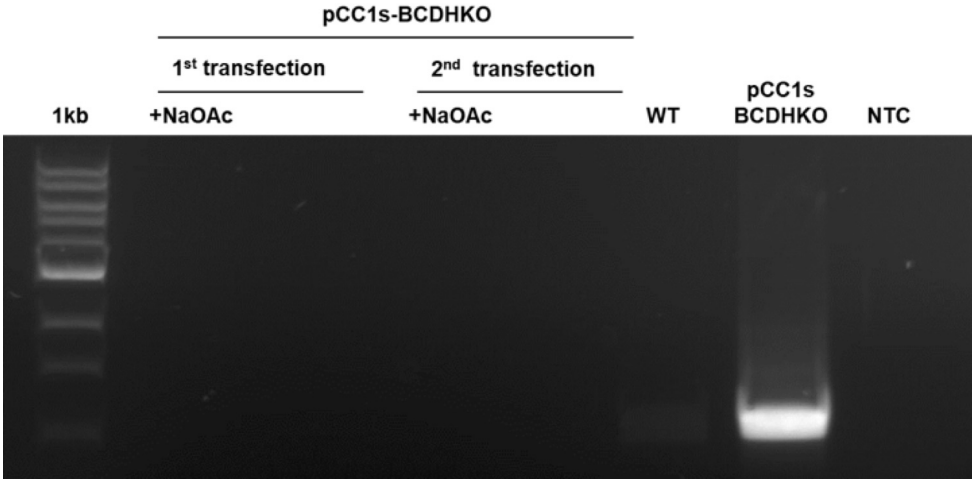

Figure 4.6: Parasite lines did not demonstrate existence of the transfection plasmid. A PCR was performed to determine whether parasites were maintaining the transfection plasmid episomally. No parasite lines demonstrate evidence of harboring the transfection plasmid. The pCC1s-BCDHKO plasmid was used as a positive control, and WT gDNA was used as a negative control.

Chapter 5

Conclusion and Future Directions

5.1 Lipoamidase in *Plasmodium*

Lipoamidase experiments were used in *P. falciparum* as a proxy for knockdown and knockout experiments due to the lack of effective/efficient tools for genetic manipulation in the parasite. Whether or not this gene would be tolerated in the parasite was unclear as expression of even the catalytically inactive mutant in *E. coli* is quite low and imparts a significant growth phenotype (Spalding and Prigge, 2009). The experiments performed here demonstrate in vivo activity and inactivation of *Plasmodium* proteins, and have revealed an important role for mitochondrial lipoylation in the parasite. The demonstrated successful use of lipoamidase in *E. coli* and in *Plasmodium* suggests that it can be used as a tunable tool for probe lipoylation in other organisms. This idea is strengthened by the fact that lipoamidase appears to work against both a biosynthetic lipoylation pathway (substrate octanoylation, followed by sulfuration) and a scavenging pathway (activation of free lipoate, followed ligation or transfer to the target protein). The experiments

performed here demonstrate the use of different promoters and active site mutations to tune this activity. Work in *E. coli* demonstrated that truncated variants can also be used to reduce lipoamidase catalytic activity. Advances in inducible and knockdown technology provide even more granularity to how much lipoylation to abrogate. Any and all combinations of these methods can be used to try to attenuate or even completely abolish *in vivo* lipoylation in other model organisms, especially in ones where the lipoylation machinery or chemistry has not been well described.

Lipoamidase expression in the mitochondrion imparts a severe growth phenotype on blood-stage *Plasmodium falciparum*. This strongly suggests that lipoylation of at least one of the mitochondrial proteins is essential for parasite survival. The fact that acetate supplementation partially rescues parasite growth suggests that mitochondrially generated acetyl-CoA is important for parasite growth. There are a number of nonexclusive explanations for why acetate supplementation does not completely restore parasite growth: conversion of acetate to acetyl-CoA could be too slow to keep up with demand, or acetate derived acetyl-CoA inefficiently trafficks to pathways that glucose-derived acetyl-CoA is used for (Cobbold et al., 2013), or the H protein is essential for a role that cannot be bypassed by acetate supplementation. Considering the difference in phenotypes observed in *P. falciparum* and *P. berghei* in the TCA knockouts discussed in Chapter 1, it may be possible that there are species-specific differences in requirements for the three lipoylated proteins found in the mitochondrion. Though it has been demonstrated that the *P. falciparum* KDH E1 is competent at performing PDH E1 catalysis (Chan et al., 2013), it is not known whether this is the case in *P. berghei*. Thus, there may be

redundancy of function in one parasite species that does not exist in another.

It would be interesting to observe lipoamidase expression in *P. berghei*. There has not been experimental evidence to confirm that *P. berghei* also scavenges lipoate for mitochondrial lipoylation, and considering the number of different phenotypes observed in related pathways (e.g. the LipB knockout resulting in increased BCDH lipoylation in *P. berghei* but not *P. falciparum* (Falkard et al., 2013; Günther et al., 2007)) it remains a formal possibility that abrogating lipoylation in the murine parasite may result in different phenotypes than that observed in *P. falciparum*. This is also somewhat complicated by the fact that metabolic flux in a mouse is not identical to that of a flask, and that includes the steady level of acetate in blood that is not present in parasite culture media. This then remains an experimentally challenging task, as specifically radiolabeling parasites in a mouse is a hard target, and so multiple experiments, such as lipoamidase expression, *ex vivo* radiolabeling, and a panoply of knockouts, would be required to determine the importance of lipoylation in *P. berghei*, including the possible immunological or other non-biochemical consequences of ablating lipoylation.

Additionally, experiments in murine models would allow us to interrogate the phenotypes of lipoamidase expression during the parasite's other stages, e.g. gametocytes, insect stages, etc. The parasite appears to take on a more auxotrophic mode as it leaves the asexual blood stage, so it would be curious to see how this affects lipoate-mediated metabolism. It is unclear whether there is sufficient lipoylated protein to support parasite development from gametocyte to sporozoites, so the necessity of lipoate salvage for the

mitochondrial proteins is in question. Some FLP-FRT experiments have been successfully performed in *Plasmodium* (Choudhary et al., 2018; Kublin et al., 2017; Lacroix et al., 2011), so setting up a stage-specific knockout could address these questions. Fragmentary information suggests that protein lipoylation is essential for parasite development during the mosquito stages. Failure of the ΔPDH E1 in *P.falciparum* to produce sporozoites demonstrates that lipoate synthesis and apicoplast metabolism are essential for mosquito stage development (Cobbold et al., 2013). Similarly, ΔKDH E1 *P. falciparum* parasites fail to form viable sporozoites (Ke et al., 2015), suggesting that both apicoplast and mitochondrial lipoate metabolism (and lipoate scavenging) may also be required for development during these stages.

5.2 Mitochondrial lipoylation is essential in *Plasmodium*

Obviously, knocking out or knocking down the lipoate attachment enzymes, LipL1 and LipL2, would strengthen the hypothesis that these are indeed the machinery responsible for mitochondrial lipoylation. The inherent problem with knock out experiments is that the genetic tools to generate conditional knockouts don't yet exist for *P. falciparum*. Thus, successful knock out of an essential gene does not result in viable parasites. Knock down methods exist, however, the limited dynamic range of these methods would not allow complete elimination of a particular protein target or its activity. A metabolic bypass was attempted in the lipoamidase experiments, but this bypass has limited effect, suggesting that complete ablation of mitochondrial

lipoylation would likely kill the parasite. As such, it would likely be that these genetic experiments would ideally be coupled to other experiments, such as the development of LipL1/LipL2 inhibitors to confirm our hypothesis, e.g. a LipL1 knockdown experiment coupled to 8-bromooctanoate treatment to see if there is a synergistic effect in inhibiting parasite growth. These experiments would have the added benefit of validating a potential new drug target for therapeutic intervention.

It is most likely the case that blood-stage *Plasmodium* parasites require mitochondrially-derived acetyl-CoA. Acetyl-CoA is a ubiquitous metabolite, serving as a metabolic intermediate or precursor for many basic metabolites, and is also one of the chief means of protein regulation. Acetyl-CoA is typically derived through the pyruvate dehydrogenase (PDH) pathway, and in eukaryotes, this activity exists in mitochondria. This is probably also the case for malaria parasites; however, the best candidate enzyme in the mitochondrion was bioinformatically described as a BCDH (branched chain amino acid dehydrogenase) involved in the metabolism of branched chain amino acids. The apicoplast resident PDH was thought to be the only bona fide PDH (Foth et al., 2004) . Work done here and elsewhere (Cobbold et al., 2013; Oppenheim et al., 2014) suggests that if the apicoplast PDH is generating acetyl-CoA during the blood stage, it is of little consequence to the parasite as a whole. Instead, the mitochondrial enzyme annotated as the BCDH appears to function as a PDH in *P. berghei* (Oppenheim et al., 2014). If this is also the case in *P. falciparum*, it provides an excellent explanation for why inhibition of this enzyme by lipoamidase is rescued by acetate supplementation.

It will be very interesting to determine the role of the H protein in the parasite mitochondrion. Research in our lab and other investigators have so far failed to knock out the H protein (Varadarajan et al., 2014) and H proteins are typically described as part of a glycine cleavage system, such as that found in *E. coli*. The genomes of malaria parasites do not appear to encode two of the other enzymes needed for glycine cleavage activity, suggesting that there is a different role for this protein in malaria parasites. LipL1 only lipoylates the H protein under relatively oxidizing conditions, suggesting that the role of the H protein is associated with oxidative conditions. One thought is that the H protein is involved in an oxidative defense mechanism similar to that recently observed for the H protein from *T. vaginalis* (Bryk et al., 2002). Pulldown of the H protein would be informative not only to see what other proteins may interact with it, but to also interrogate what modifications may exist on the lipoate moiety. As mentioned in previous chapters, it is difficult to detect lipoylated H protein in Western blots, and this could be due to a chemical modification, such as oxidation of the lipoate sulfur to sulfate or sulfone, nitrosylation, etc. Performing pulldowns under various conditions, such as high parasitemia, rings vs. schizonts, under drug pressure, etc., may help to support the oxidative defense hypothesis.

Ultimately it would also be interesting to understand why protein lipoylation in the mitochondrion of malaria parasites is gated by redox conditions. This phenomenon has not been observed in any other organisms and it may be connected to an overall response to the different redox environments that malaria parasites encounter during their overall life cycle. As mentioned in

Chapter 1, the parasite's serine hydroxymethyltransferase, which is essential for folate-based single carbon metabolism, appears to have a redox switch that controls its function. In the apicoplast, the acyl carrier protein appears to dimerize under oxidative conditions (Gallagher and Prigge, 2009). These proteins, which are located in different subcellular compartments, may help malaria parasites to respond to redox conditions by altering various metabolic pathways. Although this would be a long term project, it would be interesting to see if parasites have a global metabolic response - one program under an oxidative environment and another program for a reductive environment. It would then be interesting to overlay this paradigm against the multiplicity of host environments the parasite encounters, and what their respective redox environments contribute.

5.3 Apicoplast lipoylation is dispensable in blood-stage *Plasmodium*

It is maddening that no experiment has completely abolished lipoylation in the parasite apicoplast. In the lipoamidase experiments, it is possible that this stubbornly persistent signal is crossreactivity against the octanoylated, and not lipoylated, PDH E2 subunit. However, this does not explain why the PDH E2 subunit remains somewhat lipoylated in the octanoyl transferase knockout (ΔLipB) experiments (Falkard et al., 2013; Günther et al., 2007). The possible dual localization of LipL2 (Günther et al., 2009) is an explanation, although not a satisfactory one, to explain this phenomenon. A combination of knockouts, e.g. LipB and LipL2, may confirm or refute this analysis, although

the potential growth defect of the LipL2 knockout would have to be complemented with acetate supplementation. Knockout of the PDH E2 subunit has not yet been described, but this deletion should remove all lipoylated protein in the apicoplast; any residual signal in a ΔPDH E2 line would reveal the presence of a novel lipoylated apicoplast protein. Finally, the question of how octanoate is derived for apicoplast lipoate synthesis exposes a lack of clarity regarding parasite fatty acid synthesis. Blood stage parasites don't seem to make fatty acids and don't have a biotinylated ACC (Acetyl-CoA Carboxylase) to generate the carbon source (malonyl-CoA) for synthesis (Delli Bovi, et al *in press*).

There may be some explanation in the other pathway that involves pyruvate. Pyruvate in the apicoplast appears to have two fates: either consumption by the PDH pathway, or consumption into the isoprenoid pathway. As mentioned above, it seems that the PDH is not consuming much pyruvate, so pyruvate is likely primarily consumed in isoprenoid biogenesis. Isoprenoids are a multifunctional precursor for many products in *Plasmodium*, with likely essential roles in farnseylation (Gisselberg et al., 2017a) and geranylation (Gisselberg et al., 2017b). There is some evidence that apicomplexans may generate plant like molecules from isoprenoids, such as gibberilic acid, absicic acid, and isopentenyl adenine (Andrabi et al., 2018), which have been implicated as growth factors in *Plasmodium* and *Toxoplasma*. The different growth phenotypes observed in the *P. falciparum* LipB, PDH E1 and E3 knockouts (Cobbold et al., 2013; Günther et al., 2007; Laine et al., 2015) may be a result of disrupted pyruvate flux in the parasite: with no pyruvate exiting through

the PDH pathway, more pyruvate is available for the isoprenoid generation pathway, possibly overloading downstream products. This may then result in more secretion of these isoprenoid based molecules that affect quorum sensing in the parasite. Interestingly, some isoprenoid cursors have also been implicated in inducing increased mosquito attraction (Emami et al., 2017). It would be interesting to then see the variety of these knockouts in mosquito feeding assays both in a blood bag and through a mouse to see what contribution isoprenoid precursors have in mosquito attraction during the blood stage, and if PDH/LipB knockouts demonstrated increased mosquito feeding.

However, this does not address the function of the PDH in the blood stage of the parasite. It is still unclear how and why the parasite produces the PDH complex and activates the E2 subunit with lipoylation during the blood stage. Although it is possible that this entire arsenal of proteins is completely dispensable in the blood stage of the parasite, the varying phenotypes mentioned above and the inability to completely ablate E2 lipoylation suggest that these proteins have some blood stage role, but require more stringent interrogation outside of gross growth phenotypes.

5.4 Conclusion

Although lipoylation is an uncommon and poorly studied posttranslational modification, it is an essential basic biochemical in most forms of life. The work presented in this thesis and the work presented in the field present a coarse perspective on the role of in the parasite, where its canonical role in generating acetyl-CoA is likely conserved throughout the parasite's lifecycle

through the mitochondrion and occasionally through the apicoplast via their respective PDH's, and in generating succinyl-CoA outside of the blood-stage via the KDH. Secondary, noncanonical roles likely exist for these proteins as well, most likely in redox balance. Fully delineating these roles would help understand parasite biology better, potentially provide further drug targets, and provide greater insight to noncanonical/arcane/ancient biochemistry that has not yet been observed in other life.

5.5 References

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Chapter 6

Appendix I: Improving yield of ^{35}S lipoate

6.1 Abstract

Our lab has had previous success in generated radiolabeled lipoate, but yield of radioactive material per preparation average around 1%. Poor yield results in long exposure times, and coupling this to the short half-life of sulfur-35 make radiolabeling experiments cost prohibitive. The experiments in this project attempted to improve this yield, and ultimately, it was determined that re-use of spent bacterial media improves yield to approximately twice as much as before. Experiments are ongoing to see if yield can be improved further.

6.2 Introduction

Radiolabeled lipoate is particularly useful for demonstrating whether cells

import lipoate and for visualizing the fate of scavenged lipoate. Lipoylated proteins can also be observed by western blot with an anti-lipoate antibody, however, this method cannot discriminate between scavenged and synthesized lipoate. Unfortunately, there are no vendors who sell radiolabeled lipoate, so methods are required to synthesize it. Chemical synthesis of ^{75}Se-labeled lipoate have been described (Reed et al., 1994) but this method produces a racemic mixture of *R* and *S* lipoate, whereas cells specifically synthesize and use the R stereoisomer. In our method, we harness the ability of *E. coli* cells to synthesize *R*-^{35}S-lipoate.

Work in our lab has established a protocol to generate radiolabeled lipoate (**Figure 1**) (Allary et al., 2007). First, we express a recombinant, truncated, His-tagged *E.coli* H protein with a C59S mutation that can be lipoylated by the *E.coli* lipoylation machinery. These constructs are expressed in the B834(DE3) strain of *E. coli*, which are methionine auxotrophs, grown in in minimal media, with the only sulfur source coming from exogenously supplied methionine in order to limit sulfur metabolism. Octanoate is provided for facile attachment by the endogenous *Ec*LipB to octanoylate the H protein, and exogenous iron and radiolabeled sulfate is provided for the sulfuration of the octanoyl group to a lipoyl moiety. The bacteria also expresses an exogenous, iron sulfur cluster biosynthesis operon from *A.vinelandii* to facilitate iron sulfur cluster biogenesis to facilitate the sulfuration reaction. The protein is then affinity purified (**Figure 2**), TCA precipitated, and then acid hydrolyzed to liberate the lipoate from the purified protein. The hydrolyslate is then assayed for radioactivity to determine radioactive yield, which is typically well below 1%

of the supplemented radioactive sulfur .

Prior attempts made to improve yield include changing protein induction timing, changing the carbon source or other nutrients, and adjusting the time of acid hydrolysis. None of these changes improved yields past the average of 1%. Several other experiments were done to see if yield can be improved, and the most significant improvement in yield comes from re-using the spent media.

6.3 Results

6.3.1 Determining maximum theoretical yield

Previous work did not determine whether higher yields of lipoate could be obtained using this method. In order to determine if >1% yield was even theoretically possible, *E. coli* was grown in minimal media and in rich, TB (terrific broth) medium and protein yields were determined (**Table 1**). After acid hydrolysis, hydrolysates were supplemented with a strain of *E. coli* reliant on scavenged lipoate for optimal growth to approximate the bioavailable free lipoate from these preparations. This method was used as a surrogate for using radiolabeled sulfur due to cost considerations, and previous experiments indicate that comparative yield using radiolabeled sulfur result in a concentration of 2 nM lipoate.

Protein yields between TB and minimal media were reasonably equivalent, but the amount of bioavailable lipoate from TB when normalized to protein yield was at least forty times higher than any other experiment listed in **Table 1**. These data indicate that under optimal conditions, the recombinant protein

can be lipoylated significantly more than previously described, but only to 1% of the total protein.

6.3.2 Testing induction and incubation times and temperatures

The current protocol for purification is a stepwise induction, followed by treatment with radiolabeled sulfate and shaking at 20°C for 10 hours, followed by a 4°C incubation until the culture can be harvested (typically the next day). It was thought that this protocol might allow for more facile expression and lipoylation of the H protein.

Considering the poor lipoate yield, the temperatures and times for induction and incubation were changed to see if superior yield was possible. In addition, pseudo-anoxic incubation was also tried because of the sensitivity of iron sulfur clusters to air. Samples for each condition were taken and run on Western blots using the anti-lipoate antibody to look for relative lipoylated protein.

Results in **Figure 3** show that protein induction and lipoylation is somewhat nominal under nearly all conditions, as expression under multiple conditions show that exogenous, lipoylated H protein levels are not higher than the endogenous lipoylated proteins. Bands for the H protein appear relatively similar in lysates after 4°C induction, even though Ponceau staining shows significantly more loaded protein in the lanes with 4°C induction. This may mean that a 4°C incubation after induction may not improve, and may reduce, the yield of lipoylated protein.

6.3.3 Reusing spent media allows for two, sequential protein preps

Multiple runs suggest that *E. coli* only takes up approximately one-third of the added radiolabeled sulfur in the media. In order to test if this sulfur was still available for our use, the spent bacterial media was fully restored with minimal media components and used again for protein expression. **Figure 4** shows that re-use of bacterial media provides comparable uptake and production of lipoylated H protein. One attempt was made to reuse this media a third time, but negligible radioactivity was taken up.

6.4 Discussion

The expense, hassle and quick half-life of producing ^{35}S-labeled protein necessitates optimizing yield. Reusing the media effectively doubles the yield of the previous protocols, which is a massive improvement. However, expression under optimal conditions demonstrates that lipoylation (and not protein expression or octanoylation) is the true limiting factor in producing radiolabeled lipoate.

The purified H protein fraction typically shows up as a doublet (**Figure 2**). There are three potential products in this fraction: the unmodified H protein, the octanoylated H protein and the lipoylated H protein. SDS-PAGE might be able to resolve the charge difference between a the apoprotein with a free lysine residue, and its octanoylated (or lipoylated) species (Christensen and Cronan, 2010; Morris et al., 1995). (Christensen and Cronan, 2010; Morris et al., 1995). Considering the poor sensitivity of Coomassie staining and the poor

yield of lipoate in our reactions, it is possible that the two bands observed during purification are the apo-H protein and the octanoylated H protein. This would suggest that while the protein is readily octanoylated, the severely limiting step is the sulfuration of the octanoate to lipoate. It should be noted that it remains a formal possibility that the yield of bioactive lipoate (lipoate detectable in our bioassay) is lost primarily during the acid hydrolysis step. However, similar experiments in producing radiolabeled biotin would suggest that this is not the case (Delli-Bovi et al., 2010).

The lack of significant sulfuration may be due to poor iron or sulfur mobilization by *E. coli*, insufficient iron sulfur cluster generation by the *A. vinelandii* operon, or poor activity of the *E. coli* sulfuration enzyme, LipA. Experimentation with time and temperature suggest that these variables have limited effect on improving lipoylation, so allowing extra time for endogenous processes like iron/sulfur mobilization or cluster biogenesis does not seem to generate significantly more product. The data from **Figure 3** may suggest that the 4°C incubation may be reducing yield as signal for the lipoylated, exogenous protein is not significantly higher, even though the signal for the endogenous lipoylated proteins is. This could also be explained by increased lipoylation of the endogenous substrates as a response to the cold, or possible oxidative damage of the H protein's lipoyl moiety, reducing the amount of antibody-recognizable lipoate. In any case, it would seem that yield and time efficiency favors a 37°C induction for 4 hours with no post-induction incubation.

Multiple groups have used the *A. vinelandii* operon to produce sufficient, iron sulfur cluster using proteins (Delli-Bovi et al., 2010) for assays (Seravalli

and Ragsdale, 2008) and structural studies (Fenwick et al., 2015), suggesting that there should be sufficient iron sulfur clusters available. This points to the sulfuration step being the only likely and actionable step to improve yield of lipoylated protein. Previous work in our lab has successfully used the *P. falciparum* homolog of the *E. coli* LipA to successfully sulfurate *E.coli* substrates *in vivo*. This would suggest that expressing this homolog in the H protein expression line should improve lipoylation. Work is currently being performed to get this underway.

6.5 Methods

6.5.1 General expression protocol

E. coli H protein was expressed and purified as previously described (Allary et al., 2007). Briefly, methionine auxotroph *E.coli* B834(DE3) was transformed with plasmid expressing the truncated, His-tagged *E. coli* H protein with the C59S mutation to remove all cysteines from the coding sequence (pLZ003) and the pDB1292 plasmid expressing the *A. vinelandii* iron sulfur cluster biogenesis operon. Cultures were initially shaken at 37°C in 10 mL minimal media (58 mM K_2HPO_4, 29.7 mM NH_4Cl, 16.7 mM NaH_2PO_4, 0.83 mM $MgCl_2$, 9.5 mM citric acid) supplemented with 0.4% (w/v) glucose, 100 μM $FeCl_3$, 500 μM methionine, 50 μg/ml kanamycin and 100 μg/ml carbenecillin. Exogenous *Ec*H protein expression was induced with 0.4mM IPTG at OD=0.8, followed by supplementation to 50 μM sodium octanoate and 0.01% arabinose one hour later, and finally 2-3mCi of Na $^{35}SO_4$ one hour after that. The culture was then transferred to a 20°C incubator and shaken for 10 hours. Cultures were

pelleted and lysed in 2 mL BugBuster supplemented with 1 mg/mL lysozyme and 2.5 μg/mL DNAseI.

6.5.2 Column purification protocol

The following buffers were prepared: Buffer A (20 mM NaH_2PO_4, 20 mM K_2HPO_4, 200 mM NaCl, pH 7.5), Buffer B (20 mM NaH_2PO_4, 20 mM K_2HPO_4, pH 7.5), and Buffer C (20 mM NaH_2PO_4, 20 mM K_2HPO_4, pH 5.0).

After BugBuster lysis, the *E. coli* lysate was centrifuged at 13,000 xg, and the supernatant was loaded onto a nickel column connected to a benchtop peristaltic pump (Dynamax RP-1) and the entire purification was run at 0.6mL/minute. The column was then washed with 7.5 mL Buffer A; this fraction was collected and labeled FT1 (flowthrough 1). The column was eluted with 5 mL 10 mM imidazole in Buffer A, 2 mL 30 mM imidazole in Buffer A and 4 mL 50 mM imidazole in Buffer B. This 11 mL fraction was collected and labeled W1 (wash 1). The Q column was then connected to the nickel column output, and 5mL of 400 mM imidazole in Buffer B was added, followed by 5mL of Buffer C to transfer the nickel purified protein onto the Q column. This fraction was collected and labeled FT2 (flowthrough 2).

The nickel column was then disconnected, and the Q column was attached directly to the peristaltic pump. The Q column was then eluted into the following fractions: 3 mL Buffer C, 150 mM NaCl (E1), 1 mL Buffer C, 300 mM NaCl (E2), 1 mL Buffer C, 300 mM NaCl (E3), 1 mL Buffer C, 500 mM NaCl (E4), 1 mL Buffer C, 500 mM NaCl (E5), and 3 mL Buffer C, 500 mM NaCl (E6). The purified protein typically eluted in fractions E4 and E5, and

protein precipitated in these fractions by addition of 111 μL 100% ice-cold TCA to E4, followed by centrifugation at 4C for 5 minutes. The supernatant was removed, E5 was added to the tube, along with 111 μL 100% ice-cold TCA, followed by centrifugation at 4C for 5 minutes. Supernatant was removed, pellets were resuspended in 200 μl 5 M HCl and incubated at 95C for 4.5 hours. The hydrolysate was neutralized with 100 μL 1 M K_3PO_4, 1 μL 0.02% phenol red was added, and the solution was titrated with 10 M NaOH until phenol red transitioned from a pale yellow color to red. Samples were taken at each step for scintillation counting to observe radioactivity yield.

6.5.3 Culture modifications

To determine maximal theoretical yield, 20ml TB media was used instead of 10ml minimal media described above. All other steps remained the same, except for substitution of radiolabeled sulfate for 40 μM cold sodium sulfate, which is in large excess compared to 2mCi radiolabeled sulfate. Other attempts as demonstrated in **Table 1** include doubling the media volume, later induction, and substituting glycerol for glucose.

In order to restore spent bacterial media for re-use of radiolabeled sulfur, spent media was treated as if it were water and supplemented to the minimal media composition described above, including glucose, $FeCl_3$, methionine, kanamycin and carbenecillin. Otherwise, these cultures were subsequently treated identically to freshly prepared cultures for protein purification.

To assay the effect of induction time, temperature and incubation times on protein lipoylation, multiple identical cultures were transferred to the

various conditions as described in **Figure 3**. Pseudo-anoxic conditions were performed by tightly closing the cap on the culture tube. Samples were taken at the noted points, pelleted, and lysed by resuspension in 4X NuPAGE buffer, vortexed for one minute, and boiled for 10 minutes. Samples were resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and probed with a rabbit anti-lipoate antibodies followed by an anti-rabbit HRP secondary antibodies. The blot was treated with ECL reagents, covered with saran wrap and exposed to film.

6.5.4 Determining bioavailable lipoate

Assaying the bioavailable hydrolysate was performed as previously described (Allary et al., 2007). *E.coli* deficient in lipoate biosynthesis (KER176) and reliant on lipoate scavenging for optimal growth was used to determine the levels of bioavailable lipoate. Bacteria were grown in various, known concentrations of lipoate to draw a standard curve of absorbance at 595nm vs. concentration of lipoate. 10μl and 50μl samples of hydrolysates were used to get an average approximation of bioavailable lipoate against the standard curve.

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6.7 Figures

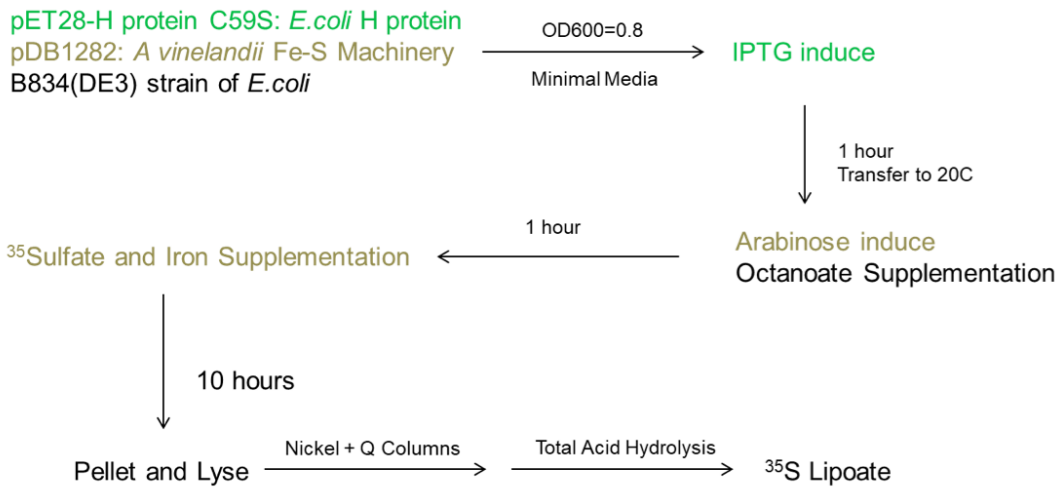

Figure 6.1: Workflow for *Ec*H protein purification The truncated his-tagged H protein is on a pET28 vector with a C59S mutation to eliminate all sulfur bearing amino acids except for the first methionine. pDB1282 is the plasmid that hold the A.vinelandii iron sulfur cluster biogenesis machinery. The B834(DE3) strain of *E.coli* is an expression competent strain that is auxotrophic for methionine to mitigate sulfur consumption for methionine. IPTG induces H protein expression, while arabinose induces the *A.vinelandii* operon. Cultures are also supplemented with excess octanoate, iron and sulfur to facilitate lipoylation. Bacteria is then pelleted and lysed, after which the supernatant is run through a nickel and Q column to purify the his-tagged H protein. The protein is then TCA precipitated, acid hydrolyzed for 4.5 hours, and then the hydrolysate is neutralized to prevent further degradation of lipoate.

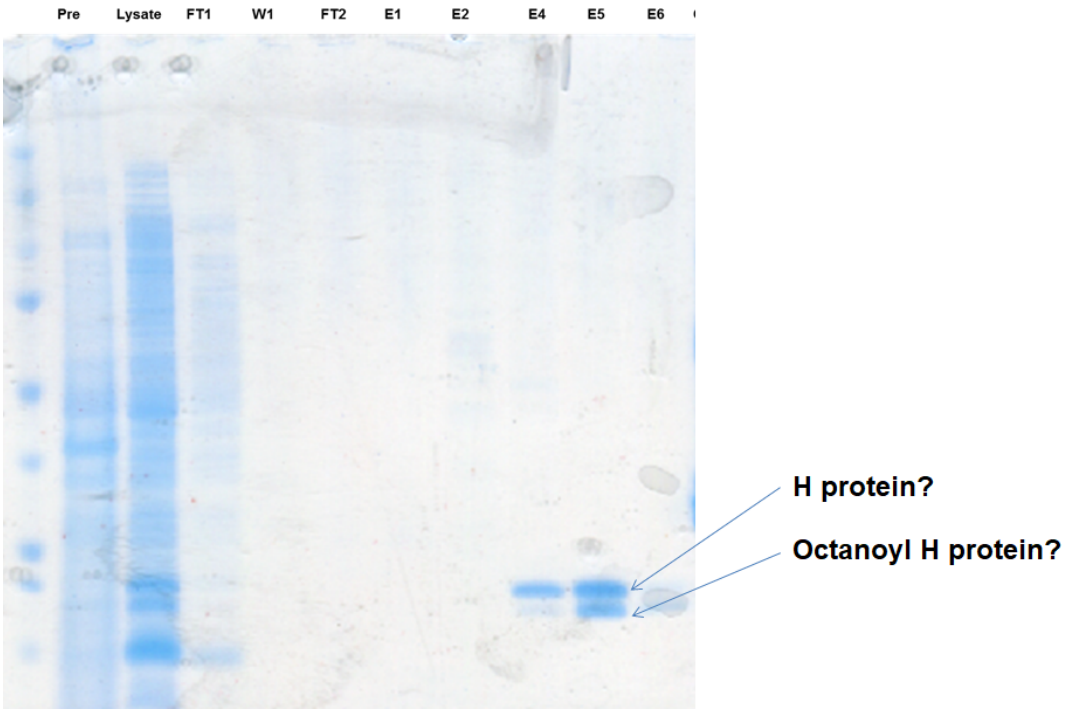

Figure 6.2: Representative gel of H protein purification Gel shows fractions from purification through the nickel and Q columns. The bands in elution fractions E4 and E5 are a doublet, that is likely the unmodified H protein and the octanoylated H protein. Coomassie staining indicates yield is reasonable, as shown in Table 1. It is worth noting that bands of similar molecular weight are apparent in the lysate, further indicating competent expression of the protein.

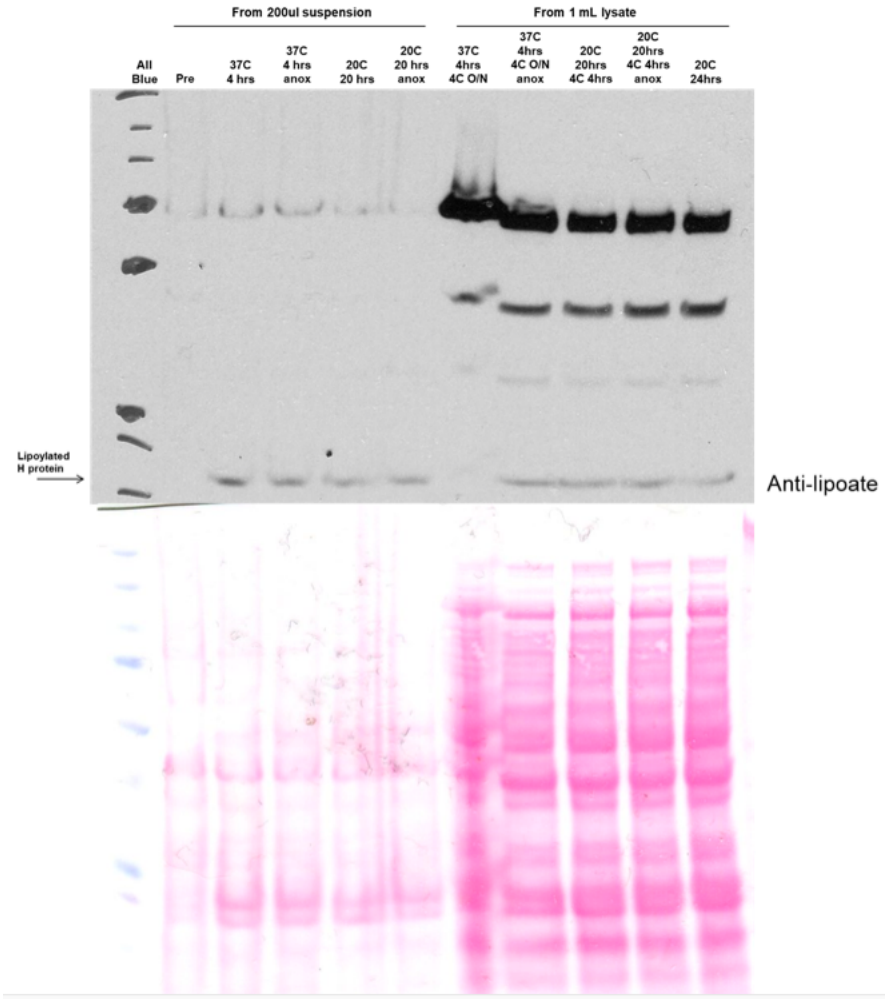

Figure 6.3: Temperature and time of induction and incubation do not generally affect H protein lipoylation Samples taken at different induction points were processed for Western blotting. The preinduction lysate demonstrates no evidence of the truncated, lipoylated, exogenous H protein. Note that amount of lipoylated protein does not change after 4°C incubation, even though signal of the endogenous lipoylated proteins is increased. This increase may be commensurate with the increased protein loaded as demonstrated by the corresponding Ponceau stain of the membrane. The higher molecular weight bands correspond to the endogenous lipoylated proteins of *E.coli*: the PDH E2, the KDH E2 and the H protein, in descending molecular weight order.

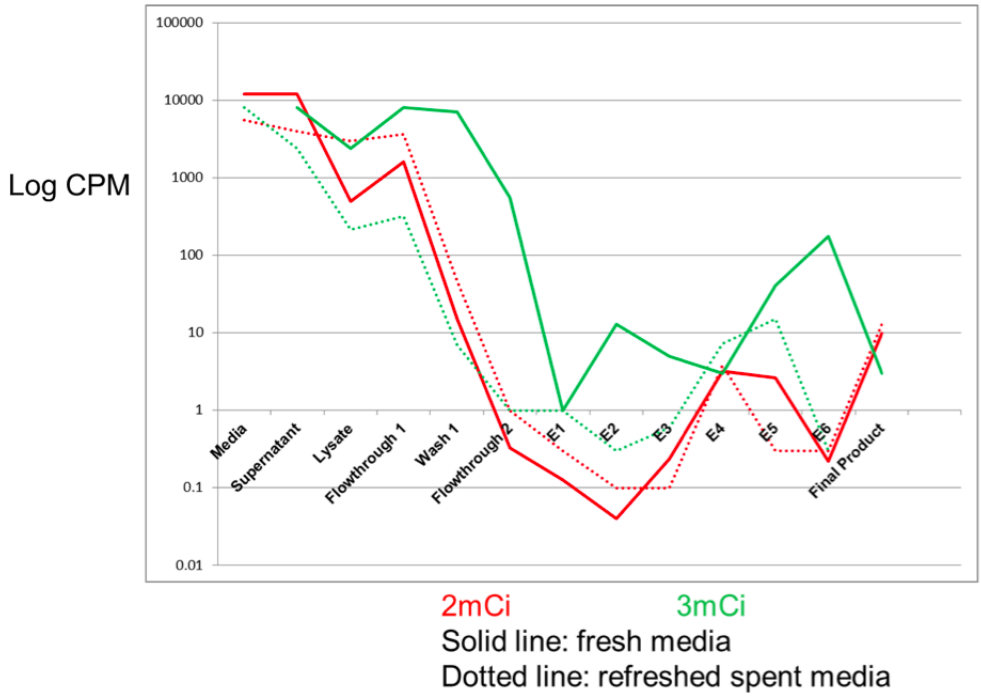

Figure 6.4: Label incorporation is very poor, but spent media can be reused Normalized graph of Log CPM of samples taken at each step of purifying the ^{35}S radiolabeled H protein. The difference between elution fractions E4 and E5 are approximately 3 logs lower than the starting radioactivity levels, indicating catastrophic loss of radioactivity. Data suggests that using spent media provides about the same amount of product as the first attempt, increasing the total yield by a factor of two. Data for 3 mCi worth of radioactivity shows some higher loss of product during purification, suggesting 2mCi is sufficient for use.

6.8 Tables

Attempt	Condition	Protein Yield	Amount of Lipoate	Proportion lipoylated	Notes
1	10mL min	200ug	25nM	0.07%	OD=0.8
2	20mL min	200ug	0.5nM	0.014%	New octanoate, iron, OD=0.9
3	20mL TB	300ug	455nM	1%	AS ABOVE, OD=1.1
4	10mL min	700ug	3nM	0.0024%	OD=0.9
5	10mL min	200ug	4nM	0.01%	Glycerol instead of glucose OD=0.8
6	10mL new min	200ug	10nM	0.025%	All new media components OD=0.8

Table 6.1: Use of TB media shows maximum lipoylation conditions are still very low Various attempts to improve yield include more media volume, changing media components and OD at induction. Tests suggest that initial conditions are likely optimal for minimal media. Insufficient lipoylation in media that provides heavy excess of nutrients (TB media) suggests the bottleneck for producing lipoylated proteins is not protein production, but lipoylation.

Chapter 7

Appendix II

7.1 Host Biotin is Required for Liver Stage Development in Malaria Parasites

Contribution includes Table 1, Table 2, Supplementary Table 1, and Supplementary Figure 8

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Host biotin is required for liver stage development in malaria parasites

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Acetyl-CoA carboxylase (ACC) is a biotin-dependent enzyme that is the target of several classes of herbicides. Malaria parasites contain a plant-like ACC, and this is the only protein predicted to be biotinylated in the parasite. We found that ACC is expressed in the apicoplast organelle in liver- and blood-stage malaria parasites; however, it is activated through biotinylation only in the liver stages. Consistent with this observation, deletion of the biotin ligase responsible for ACC biotinylation does not impede blood-stage growth, but results in late liver-stage developmental defects. Biotin depletion increases the severity of the developmental defects, demonstrating that parasite and host biotin metabolism are required for normal liver-stage progression. This finding may link the development of liver-stage malaria parasites to the nutritional status of the host, as neither the parasite nor the human host can synthesize biotin.

acetyl-CoA carboxylase | apicoplast | holocarboxylase synthetase | biotin ligase | *Plasmodium*

Biotin is an essential metabolite in all known free-living organisms. It functions as a CO_2 carrier in carboxylation and decarboxylation reactions catalyzed by a small family of enzymes, the biotin carboxylases (1). Biotin carboxylases participate in central metabolic processes such as gluconeogenesis, lipogenesis, amino acid metabolism, and energy transduction (2). Bacteria, plants, and some fungi can synthesize biotin de novo from a pimeloyl-CoA precursor, but other organisms acquire biotin from their diet or environment (3). Regardless of how biotin is acquired, a dedicated ATP-dependent biotin ligase, or holocarboxylase synthetase (HCS), is required to catalyze the covalent attachment of biotin to a specific lysine in the target carboxylase (4).

Acetyl-CoA carboxylase (ACC) is the only protein predicted to be biotinylated in the parasite (5). ACC enzymes, the most common member of the biotin carboxylase family, are found in most living organisms and catalyze the first and rate-limiting step in fatty acid synthesis (FAS) (6). The *Plasmodium* ACC resembles the homomeric ACC found in the chloroplasts of grasses in that it contains three functional domains expressed as a single large polypeptide (7), and localizes to a plastid organelle called the apicoplast (8). The ACC orthologs that function in the plant chloroplast are common targets for herbicides (1). Some of these inhibitors have been tested against blood-stage malaria parasites (9), but it was shown that any growth inhibition observed with these compounds is the result of off-target effects, as *Plasmodium falciparum* ACC can be disrupted (8). ACC has two catalytic domains: a biotin carboxylase (BC) domain that transfers CO_2 from bicarbonate to biotin and a carboxyltransferase (CT) domain that transfers CO_2 from biotin to acetyl-CoA to generate malonyl-CoA. A third domain, known as biotin carboxyl carrier protein (BCCP), contains the conserved lysine to which biotin is attached. Biotin functions as a swinging arm to transfer the CO_2 moiety between the active sites of the BC and CT domains (1). The enzymatic domains of *Plasmodium* ACC have not yet been studied, but it has been shown that the BCCP domain of *P. falciparum* is biotinylated when expressed in *Escherichia coli* (10).

Malaria parasites contain a type II FAS (FASII) pathway in the apicoplast (11) that relies on malonyl-CoA as the two-carbon subunit for fatty acid elongation (12). Gene KOs of FASII pathway enzymes in the rodent parasites *Plasmodium yoelii* and *Plasmodium berghei* demonstrated that the FASII pathway is required for normal liver-stage development, but not for blood- or mosquito-stage development (13, 14). Thus, ACC and biotin should be required in the liver stages to provide malonyl-CoA for downstream FASII pathway enzymes. Consistent with this idea, pyruvate dehydrogenase, which produces acetyl-CoA, the substrate of ACC, has also been shown to be critical for liver-stage development in *P. yoelii* (15).

Biotin metabolism may also be important for other stages of parasite development. Malaria parasites appear to encode enzymes comprising a fatty acid elongation (ELO) pathway similar to that described in the apicomplexan parasite *Toxoplasma gondii* (16). As is the case for typical ELO pathways (17), enzymes of the *T. gondii* ELO pathway are associated with the ER membrane and use malonyl-CoA as a substrate (16). *P. falciparum* parasites lacking a functional FASII pathway can still elongate fatty acids, possibly because of the activity of the ELO pathway (14). If malonyl-CoA can cross the apicoplast membranes, ELO as well as FASII could depend on ACC activity and biotin metabolism.

Perhaps the most unusual feature of biotin metabolism in malaria parasites is the presence of two HCS paralogs encoded

Significance

Malaria parasites require certain host nutrients for growth and survival. In this project, we examined the role of the human vitamin biotin in all stages of the malaria life cycle. We cultured blood- and liver-stage malaria parasites in the absence of biotin and found that, whereas blood-stage replication was unaffected, liver-stage parasites deprived of biotin were no longer capable of establishing a blood-stage infection. Interestingly, biotin depletion resulted in more severe developmental defects than the genetic disruption of parasite biotin metabolism. This finding suggests that host biotin metabolism also contributes to parasite development. Because neither the parasite nor the human host can synthesize biotin, parasite infectivity may be affected by the nutritional status of the host.

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in the genomes of *Plasmodium* species. Plant cells also contain two HCS paralogs (18), and HCS activity is partitioned among the three compartments in which biotin-dependent carboxylases are found: the mitochondria, chloroplasts, and cytosol (19, 20). In *Arabidopsis thaliana*, alternative splicing governs the production of multiple *At*HCS1 isoforms that are targeted to different compartments in the cell (21). Multiple isoforms of *At*HCS2 are also produced, but these do not appear to have HCS activity, and deletion of the *At*HCS2 gene has no observable phenotype in *A. thaliana* (21). We sought to determine the role of biotin across the malaria life cycle, including how biotin is acquired, the activity and localization of the biotin ligases, and the consequence of disrupting biotin metabolism in malaria parasites.

Results

ACC Is Located in the Apicoplast in Liver and Blood Stages but Is Not Biotinylated During the Blood Stages. ACC is the only predicted biotin-dependent enzyme in the *Plasmodium* genome. ACC has previously been localized to the apicoplast in blood-stage *P. falciparum* (8), but the localization has not been confirmed in the other stages of the parasite life cycle or in other *Plasmodium* species. We localized ACC in liver-stage *P. berghei* through immunofluorescence microscopy of infected HepG2 human liver cells by using antibodies directed against the BCCP domain of *Pf*ACC (10). We found that BCCP colocalizes with an apicoplast marker, the acyl carrier protein (ACP), indicating that endogenous *Pb*ACC resides in the apicoplast in liver-stage parasites (Fig. 1*A*).

ACC enzymes are only active when covalently modified by biotin. To determine whether *Pb*ACC could be biotinylated in the liver stages, we performed immunofluorescence microscopy of HepG2 cells infected with *P. berghei* by using antibodies against biotin. We observed that biotin colocalizes with the apicoplast marker ACP, indicating that biotinylated proteins reside in the apicoplast in liver-stage malaria parasites (Fig. 1*B*).

We next looked at ACC expression and biotinylation in blood-stage *P. berghei* by using immunofluorescence analysis with antibodies specific to BCCP and biotin. We found that *Pb*ACC is expressed in the apicoplast in blood-stage parasites (Fig. 1*C*), but we were unable to detect any biotinylated proteins in the blood stages (Fig. 1*D*). Thus, ACC is expressed in the apicoplast in blood- and liver-stage malaria parasites, but appears to be biotinylated only in the liver stages.

To determine whether endogenous ACC is biotinylated in blood-stage *P. falciparum*, we performed immunofluorescence analysis of Dd2^{attB} parasites by using antibodies specific to BCCP and biotin. We could detect ACC in the apicoplast, and, in some experiments, ACC protein appeared to be proximal to the apicoplast as well (Fig. 1*E*). This phenomenon was also observed in *P. berghei* and may reflect slow kinetics of trafficking such a large protein (~3,000 aa). We could not detect any biotinylated protein in *P. falciparum* because there was no significant signal above the background autofluorescence observed for uninfected red blood cells (Fig. 1*F*). These results indicate that, as in *P. berghei*, *Pf*ACC is expressed but not biotinylated in the blood stages, and raise the possibility that ACC activity is regulated through stage-specific biotinylation.

To investigate the requirements for ACC trafficking to the apicoplast, we expressed the first 115 residues of *Pf*ACC (containing the putative targeting domain) fused to a C-terminal GFP tag in Dd2^{attB} parasites. We found that ACC-GFP localizes to an elongated, branched organelle distinct from the mitochondrion, which is consistent with apicoplast morphology (*SI Appendix*, Fig. S1*A*), and colocalizes with the apicoplast marker ACP (*SI Appendix*, Fig. S1*B*). We also observed that the fusion protein was processed normally after import into the apicoplast (*SI Appendix*, Fig. S1*C*). Taken together, these results demonstrate that the ACC leader peptide is sufficient to direct the protein to the apicoplast in blood-stage *P. falciparum*.

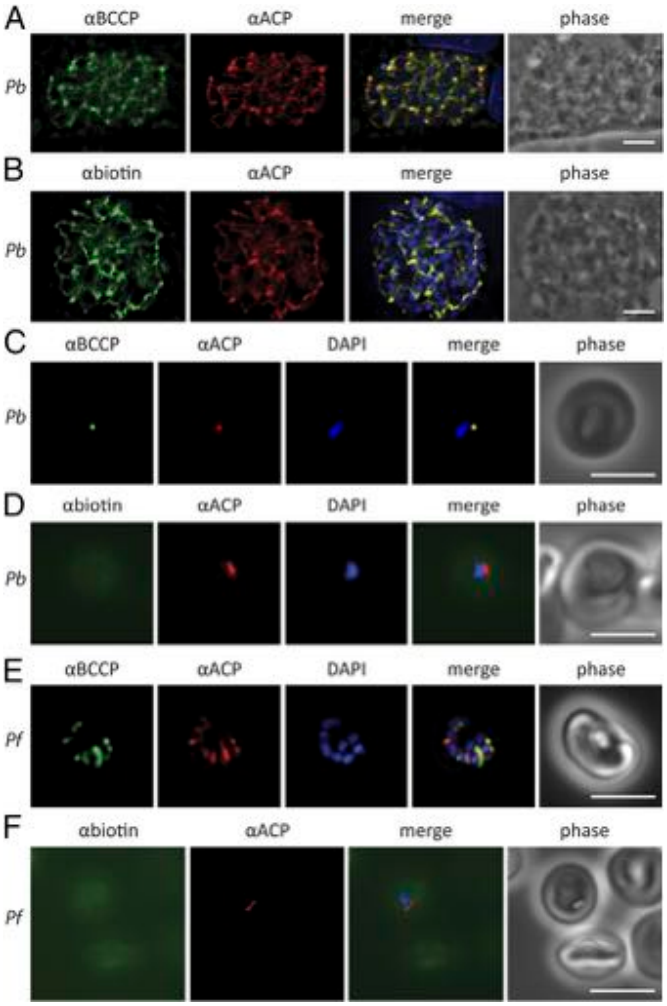

Fig. 1. ACC is expressed in the apicoplast and is biotinylated only in liver-stage parasites. (*A*) Immunofluorescence analysis of HepG2 cells at 48 hpi with *P. berghei* showing colocalization of *Pb*ACC, labeled with antibodies specific to the BCCP domain of ACC (αBCCP), with the apicoplast marker αACP. The nuclei are labeled with DAPI. (*B*) Immunofluorescence analysis similar to *A* showing colocalization of biotinylated proteins (αbiotin) with the apicoplast marker αACP. (*C*) Immunofluorescence analysis of blood-stage *P. berghei* showing colocalization of *Pb*ACC (αBCCP) with the apicoplast marker αACP. (*D*) Immunofluorescence analysis of blood-stage *P. berghei* showing no detectable biotinylated protein (αbiotin). (*E*) Immunofluorescence analysis of blood-stage *P. falciparum* showing colocalization of *Pf*ACC (αBCCP) with the apicoplast marker αACP. (*F*) Immunofluorescence analysis of blood-stage *P. falciparum* showing no detectable biotinylated protein (αbiotin) compared with an uninfected control. (Scale bars: 5 μm.)

HCS1 and HCS2 Localize Primarily to the Cytosol. We identified two biotin ligase paralogs (HCS1 and HCS2) in the *Plasmodium* genome. At least one of these enzymes should be responsible for catalyzing the biotinylation of *Pb*ACC; however, neither was predicted to localize to the apicoplast by targeting algorithms. To determine the subcellular location of the two biotin ligases in *P. berghei*, we tagged both genes with GFP through single cross-over recombination (*SI Appendix*, Fig. S2). Immunofluorescence analysis demonstrated that the GFP-tagged proteins are not predominantly located in the apicoplast in liver-stage or blood-stage parasites (*SI Appendix*, Fig. S3). Because of its size, the GFP tag may alter protein trafficking and subcellular location. To address this possibility, we generated HA-tagged parasite lines through double cross-over recombination (*SI Appendix*, Fig. S4). Consistent with the results from the GFP-tagged lines, the

HA-tagged proteins do not have obvious apicoplast colocalization (Fig. 2). This was true at different time points during liver-stage development (24, 48, and 56 h) and during blood-stage development (Fig. 2*C*).

We also localized the two biotin ligase paralogs in blood-stage *P. falciparum*. We overexpressed a second copy of each ligase as a GFP fusion protein in Dd2^{attB} parasites and characterized these lines by using fluorescence microscopy of live cells labeled with DAPI. We found that both fusion proteins primarily localize to the cytosol (Fig. 3 *A* and *B*). We also performed Western blot analysis on whole-cell lysate from these parasites by using antibodies against GFP and detected a band of the correct mass for the full-length fusion protein in both biotin ligase overexpression lines (Fig. 3 *C* and *D*). Interestingly, *Pf*HCS2-GFP was more difficult to detect than *Pf*HCS1-GFP despite the fact that the expression of both proteins was driven by the same regulatory elements. This may be a result of a difference in protein stability between the two ligases. Together, these results demonstrate that the two biotin ligases are primarily cytosolic in the liver and blood stages of the parasite life cycle. As biotinylated proteins are located in the apicoplast of liver-stage parasites, this raises the question of whether the biotin ligase paralogs are active ligases and are responsible for protein biotinylation in the parasite.

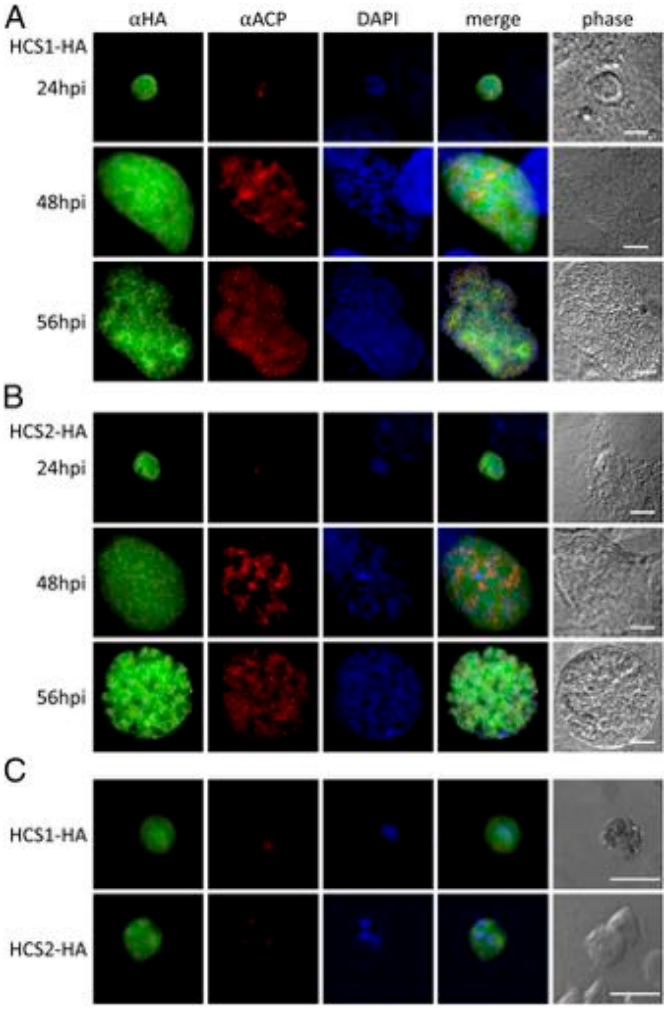

Fig. 2. HCS1 and HCS2 localize primarily to the cytosol in *P. berghei*. (*A*) Immunofluorescence analysis of HepG2 cells 24, 48, and 56 hpi with *P. berghei* parasites in which *Pb*HCS1 was tagged with an HA tag through double cross-over recombination. The tagged proteins are labeled with specific antibodies (αHA), the apicoplast is labeled with αACP, and the nuclei are labeled with DAPI. (*B*) Immunofluorescence analysis similar to that shown for *A* using *P. berghei* parasites in which *Pb*HCS2 was tagged with an HA tag. (*C*) Immunofluorescence analysis of blood-stage parasites from the transgenic lines shown in *A* and *B*. (Scale bars: 5 μm.)

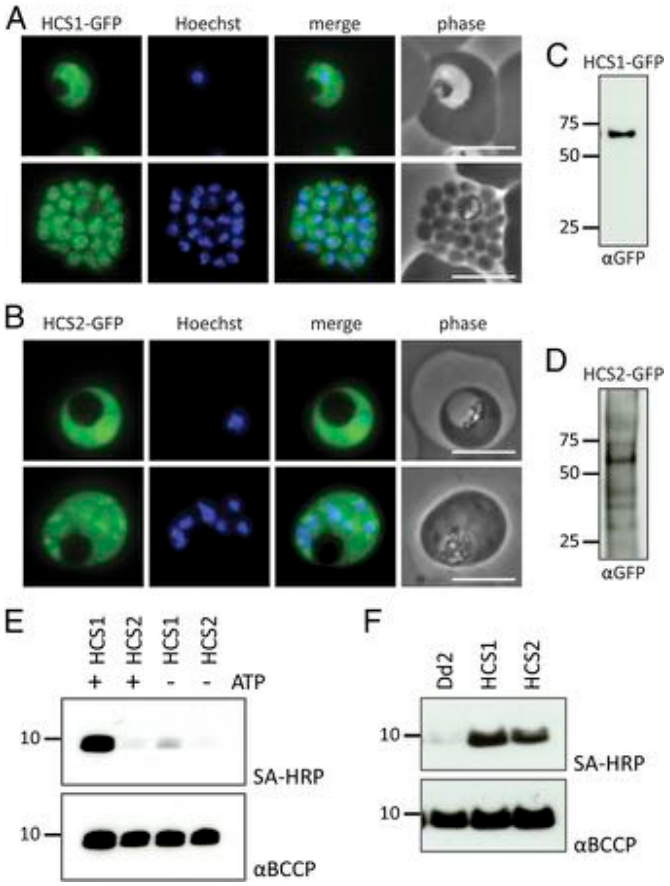

Fig. 3. *Pf*HCS1 and *Pf*HCS2 are active biotin ligases. (*A* and *B*) Epifluorescence images of live *P. falciparum* blood-stage parasites expressing full-length *Pf*HCS1 fused to GFP (HCS1-GFP) or *Pf*HCS2 fused to GFP (HCS2-GFP). The parasites were stained with Hoechst to identify nuclei. GFP fluorescence localizes to the cytosol in trophozoite (*Top*) and schizont (*Bottom*) parasites. (Scale bars: 5 μm.) (*C* and *D*) Western blot analysis of lysate from the transgenic parasites shown in *A* and *B* using antibodies against GFP (αGFP) showing a dominant band that corresponds to the expected size of the full-length fusion protein. (*E*) Recombinant *Pf*HCS1 and *Pf*HCS2 were expressed in *E. coli* and purified by affinity chromatography, and their activity was tested by using an in vitro biotin ligase assay. *Pf*HCS1, but not *Pf*HCS2, biotinylated *Pf*BCCP in vitro in an ATP-dependent reaction. (*F*) The activities of *Pf*HCS1 and *Pf*HCS2 were also tested in a cell-free assay. Lysates from parasites overexpressing the two ligases (shown in *A* and *B*), and a parental control, were incubated with purified BCCP under reaction conditions similar to *E*. *Pf*HCS1 and *Pf*HCS2 exhibit biotin ligase activity in this assay.

***Pf*HCS1 and *Pf*HCS2 Are Active Biotin Ligases.** To assess biotin ligase activity for *Pf*HCS1 and *Pf*HCS2, we first tested the activity of the two ligases in vitro. Recombinant *Pf*HCS1 and *Pf*HCS2 were expressed in *E. coli* and purified by affinity chromatography. The BCCP domain of *Pf*ACC was also expressed and purified as a biotinylation substrate. We found that overexpression of *Pf*BCCP in *E. coli* results almost exclusively in apoprotein with less than 1% of the purified *Pf*BCCP biotinylated. We tested the reactivity of each ligase toward *Pf*BCCP in vitro and assessed *Pf*BCCP biotinylation through affinity blotting with streptavidin-HRP followed by Western blotting with antibodies specific to *Pf*BCCP. We found that *Pf*HCS1, but not *Pf*HCS2, biotinylated *Pf*BCCP in an ATP-dependent reaction (Fig. 3*E*). The very low signal in the absence

of ATP is probably the result of a small amount of biotinyl-AMP copurified with *Pf*HCS1.

In addition, we performed streptavidin affinity-blot analysis of whole parasite lysate and observed a dramatic increase in total protein biotinylation in the *Pf*HCS1 overexpression line (generated as described here earlier), which was not present in the *Pf*HCS2 line or a parental control (*SI Appendix*, Fig. S5). This indicates that *Pf*HCS1 has unusually lax substrate specificity, likely because of the release of the biotinyl-AMP conjugate, which reacts spontaneously with proteins when it is free in solution.

In a final approach, we performed cell-free assays with parasite lysate. Whole-cell extract was generated from parasites overexpressing *Pf*HCS1 or *Pf*HCS2, as well as the parental control, and the reactivity of these extracts toward recombinant *Pf*BCCP was tested by using conditions similar to the in vitro biotinylation assay. In this assay, both ligases were active against *Pf*BCCP (Fig. 3*F*), although *Pf*HCS2 activity was slightly, but consistently, weaker than *Pf*HCS1 in each repetition of this assay. The low level of biotinylation observed in the parental control is likely a result of endogenous biotin ligase activity. Taken together, these results show that malaria parasites express two functional biotin ligases, which represents an unusual example of an organism that contains two active biotin ligases. The fact that we observed activity for *Pf*HCS2 only in the cell-free assay may be because *Pf*HCS2 folds correctly only when expressed in *P. falciparum* or because this enzyme requires a binding partner not present in *E. coli*.

Robust Biotinylation Activity Can Be Detected in the Cytosol of Blood-Stage Parasites. To determine whether blood-stage parasites are capable of biotinylating apicoplast proteins, we used the BCCP domain of *Pf*ACC as a genetic probe for biotinylation activity in *P. falciparum*. We expressed *Pf*BCCP with a C-terminal GFP tag in Dd2^{attB} parasites. In the absence of any targeting sequence, the BCCP-GFP fusion protein would be expressed in the cytosol. We also expressed BCCP-GFP with an N-terminal apicoplast-targeting peptide, the first 55 residues of *Pf*ACP. We generated two constructs for each protein, one in which expression was driven by the strong calmodulin (CaM) promoter, and one in which expression was driven by the moderate ribosomal protein L2 (RL2) promoter (22). We were able to generate transgenic parasites from three of the constructs: the two cytosolic constructs and the apicoplast-localized protein with the RL2 promoter. The final construct failed perhaps because overexpression of apicoplast proteins by the CaM promoter is poorly tolerated—a phenomenon we have observed before (23). We first verified that the GFP fusion proteins were correctly localized in each line by using live fluorescence microscopy (Fig. 4 *A*–*C*) and immunofluorescence analysis (*SI Appendix*, Fig. S6). We then analyzed whole-cell lysate from the transgenic parasites by using affinity blotting with streptavidin-HRP and Western blotting with antibodies specific to GFP. We observed robust biotinylation of *Pf*BCCP when it was targeted to the cytosol, but could not detect any biotinylation activity in the apicoplast in blood-stage parasites (Fig. 4 *D*–*F*). The lack of biotinylation activity in the apicoplast agrees with the biotin ligase localization data, and eliminates the possibility of a cryptic or noncanonical biotinylation mechanism in the apicoplast. This also supports the conclusion that ACC is not biotinylated, and thus inactive, in parasite blood stages.

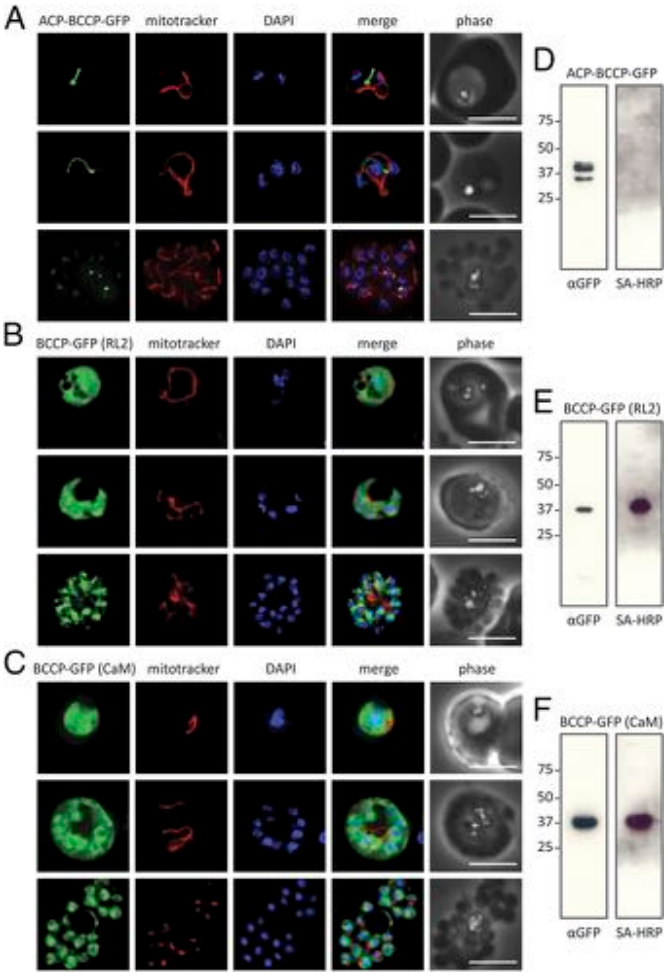

Fig. 4. Robust biotinylation activity can be detected in the cytosol but not the apicoplast of blood-stage *P. falciparum*. (*A*) The biotinylation domain (BCCP) from *Pf*ACC was expressed as a GFP fusion protein in the apicoplast of blood-stage *P. falciparum* by using the apicoplast targeting domain from *Pf*ACP (ACP-BCCP-GFP) under control of the ribosomal L2 (RL2) promoter. Localization was verified by epifluorescence microscopy in late ring (*Top*), trophozoite (*Middle*), and schizont (*Bottom*) parasites. Parasites were stained with MitoTracker to identify the mitochondrion and DAPI to identify nuclei. (*B*) BCCP was expressed as a GFP fusion protein in the cytosol (BCCP-GFP) under control of the ribosomal L2 (RL2) promoter, and localization was verified by epifluorescence microscopy as in *A*. (*C*) BCCP was expressed as a GFP fusion protein in the cytosol (BCCP-GFP) under control of the stronger CaM promoter, and localization was verified by epifluorescence microscopy as in *A*. (*D*–*F*) Whole-cell lysates from the parasite lines shown in *A*–*C* were analyzed by Western blotting with antibodies against GFP and affinity blotting with streptavidin-HRP. BCCP is biotinylated in the cytosol but is not biotinylated when expressed in the apicoplast. (Scale bars: 5 μm.)

Biotin Is Not Essential for the Survival of Blood-Stage Parasites. It has been previously demonstrated that the FASII pathway, which should be dependent on biotin, is important for the liver stages of the parasite lifecycle (13, 14). However, it is not known whether biotin is essential for parasite survival in the blood or mosquito stages. Geary et al. (24) treated blood-stage parasites with 0.2 mg/mL avidin and found that it had no effect on parasite growth in culture, but these experiments were inconclusive because they did not test whether avidin was able to permeate erythrocytes or measure the concentration of intraerythrocytic biotin stores. To determine whether blood-stage *P. falciparum* is auxotrophic for biotin, we measured parasite growth in biotin-depleted medium. The medium was prepared from biotin-free RPMI supplemented with the serum substitute AlbuMAX II. We tested the growth of Dd2^{attB} parasites as well as the three lines overexpressing the BCCP domain of *Pf*ACC (generated as described here earlier). To confirm that we had completely removed biotin from the cultures, we used *Pf*BCCP as a sentinel protein to detect intracellular biotin. It should also function as a biotin sink to further sensitize parasites to biotin deprivation because it would compete with endogenous substrates for any trace amounts of biotin that remained. We found that the presence or absence of biotin in the media made no significant difference in parasite growth (Fig. 5*A*). On the eighth

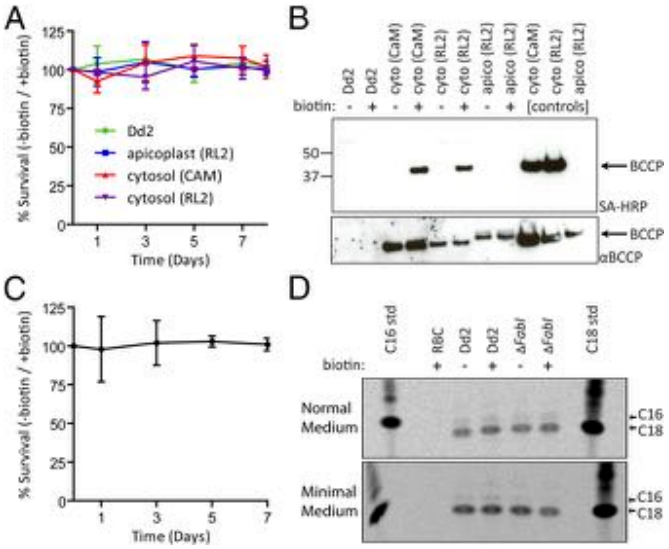

Fig. 5. Biotin is not essential for survival of blood-stage *P. falciparum*. (*A*) Blood-stage *P. falciparum* cultures were maintained for 8 d in media with or without biotin. Four parasite lines were used: the parental control (Dd2), parasites overexpressing BCCP in the apicoplast under the ribosomal L2 (RL2) promoter, and parasites overexpressing BCCP in the cytosol under the RL2 promoter or the stronger CaM promoter. Error bars represent SEM of three biological replicates. (*B*) On day 8 of the growth assay shown in *A*, parasites were harvested and biotinylation levels were assessed by streptavidin-HRP affinity blotting, followed by Western blotting with αBCCP antibodies. There was no detectable biotinylated BCCP under biotin-free conditions, indicating that biotin was completely depleted in these cultures. Controls were parasites cultured in complete medium. (*C*) Blood-stage parasites (Dd2) were cultured with or without biotin for 7 d in media containing lipid-free BSA reconstituted with 30 μM palmitic acid and 30 μM oleic acid. Error bars represent SEM of three biological replicates. (*D*) TLC of parasites labeled with ^{14}C-acetate showing incorporation into C16 and C18 fatty acids. This activity was seen in WT and FASII KO parasites, and was independent of biotin. Similar results were obtained when this experiment was repeated (*Bottom*) using the minimal fatty acid medium described in *C*. No activity was seen with the negative control, uninfected red blood cells (RBC).

day of the growth assay, parasites were isolated, and whole-cell extract was analyzed by affinity blotting with streptavidin-HRP followed by Western blotting with antibodies specific to *Pf*BCCP. There was no detectable biotinylated *Pf*BCCP in the cultures grown under biotin-free conditions, indicating that biotin was completely depleted from the medium (Fig. 5*B*). We therefore concluded that biotin is not essential for blood-stage replication in *P. falciparum*. In addition, these data show that parasites scavenge biotin rather than synthesize it, as cytosolic *Pf*BCCP is biotinylated only when biotin is added to the medium.

It has previously been shown that blood-stage malaria parasites have the ability to elongate fatty acids taken up from the medium (25). In addition, it has been demonstrated that parasites lacking a functional FASII pathway can still incorporate radiolabeled acetate into fatty acid species, most likely because of the activity of the ELO pathway (14). Based on these results, we tested whether parasites can survive without biotin under limited fatty acid culture conditions. We cultured parasites in serum-free medium supplemented with lipid-free BSA reconstituted with only palmitic acid ($C_{16:0}$) and oleic acid ($C_{18:1}$), which are the minimal requirements for parasite growth (25). Parasites cultured with or without biotin were maintained in parallel, and biotin made no difference in the growth rates of the parasites (Fig. 5*C*). These results demonstrate that biotin is not required for blood-stage parasite growth, even under conditions in which the FASII and ELO pathways would be needed the most.

Biotinylated Proteins Cannot Be Detected in Blood-Stage Parasites. Thus far, we have been unable to show any biotin-dependent phenotype in blood-stage parasites. This raises the question of whether there are any biotinylated proteins in the blood stages. We were unable to detect any signal above background in immunofluorescence analysis of blood-stage *P. falciparum* (Fig. 1*F*) or *P. berghei* (Fig. 1*D*) by using antibodies specific to biotin. Similarly, we were unable to detect biotinylated proteins in the blood stages by using streptavidin-FITC (*SI Appendix*, Fig. S7*A*). Because these data do not rule out the possibility that there are biotinylated proteins present at a level below the limit of detection by microscopy, we used more sensitive techniques to probe this question further. In radiolabel uptake experiments with blood-stage *P. falciparum*, we did not detect incorporation of [^{3}H]biotin or [^{35}S]biotin into cellular proteins (*SI Appendix*, Fig. S7*B*), even though we have shown that the parasites are capable of scavenging and incorporating exogenous biotin (Fig. 5*B*). In a final approach, we performed affinity-blot analysis of parasite lysate by using streptavidin-HRP. Although we detected biotinylated proteins in some of the blots, these bands can often be detected in samples of uninfected red blood cells (*SI Appendix*, Fig. S7*C*). These three proteins correspond in mass to the human biotin carboxylases methyl-crotonyl-CoA carboxylase (73.7 kDa), pyruvate carboxylase (127.2 kDa), and the ACC isoforms (~268.5 kDa). Although there are no biotin carboxylases expressed in mature red blood cells, they are expressed in reticulocytes and leukocytes, which can be present in small amounts as contaminants in our culture system. Thus, if blood-stage malaria parasites contain any biotinylated proteins, these proteins are much less abundant than the trace levels of human proteins in our culture system.

Biotin- and FASII-Independent Fatty Acid Elongation Activity Is Present in Blood-Stage *P. falciparum*. The growth of blood-stage *P. falciparum* parasites does not require biotin or ACC (8), leading us to the hypothesis that the ELO pathway may not rely on malonyl-CoA produced by ACC. We therefore tested whether ELO activity could be detected under biotin-free conditions. Synchronized ring-stage parasites were incubated with [^{14}C]-acetate with or without biotin, after which free fatty acids were extracted and analyzed by thin-layer chromatography (TLC). To ensure we were assessing ELO activity, we compared WT (i.e., Dd2) parasites with FASII-KO (*ΔFabI*) parasites (14). We detected radiolabeled C_{16} and C_{18} fatty acids in both parasite lines cultured with or without biotin (Fig. 5*D*). Similar results were obtained in minimal fatty acid culture medium (Fig. 5*D*), conditions previously shown to elevate FASII activity (26). Thus, blood-stage parasites are capable of elongating fatty acids independent of FASII and biotin.

Neither *Pb*HCS1 Nor *Pb*HCS2 Is Required for Blood-Stage Replication or Mosquito-Stage Development. To determine which biotin ligase is responsible for ACC biotinylation, both enzymes were knocked out in *P. berghei* ANKA parasites. The KO constructs were designed by using the pDEF-hDHFR plasmid, which facilitates double cross-over homologous recombination, resulting in excision of the entire ORF of the target gene (*SI Appendix*, Fig. S8 *A* and *C*). Correct integration of the hDHFR cassette and loss of *HCS1* and *HCS2* were verified in independent clones by diagnostic PCR (*SI Appendix*, Fig. S8 *B* and *D*). The viability of both KO strains demonstrated that neither gene is essential for survival in the blood stages. To characterize development through the mosquito stages, WT and *ΔHCS1* or *ΔHCS2* gametocytes were fed to *Anopheles stephensi* mosquitoes, and infection levels were measured at different time points. Mosquito midgut oocysts were visualized 10 d after feeding, midgut sporozoites were counted 12 and 14 d after feeding, and salivary-gland sporozoites were counted between 16 and 20 d after feeding. Throughout mosquito stage development, *ΔHCS1* and *ΔHCS2* parasites exhibited no defects in oocyst formation, sporozoite development, or sporozoite

invasion of the salivary glands compared with WT controls (*SI Appendix*, Fig. S9).

Loss of *Pb*HCS1 Affects Early Liver-Stage Development. We next evaluated in vitro liver-stage growth and morphology of *ΔHCS1* and *ΔHCS2* parasites by using immunofluorescence analysis of infected HepG2 cells at different time points during liver-stage development. Early liver-stage morphology was visualized by using mAb 3D11, which recognizes the circumsporozoite protein (CSP) on the parasite plasma membrane (27), at 24 and 48 h post infection (hpi; *SI Appendix*, Fig. S10 *A* and *B*). We also used antibodies specific for a parasitophorous vacuole protein, UIS4 (28), at 48 hpi (*SI Appendix*, Fig. S10*C*). At 24 hpi, *ΔHCS1* parasites were an average of 3 μm^2 smaller than *ΔHCS2* parasites or WT controls (*SI Appendix*, Fig. S10*D*). At 48 hpi, *ΔHCS1* parasites were an average of 53 μm^2 smaller than WT parasites (*SI Appendix*, Fig. S10*E*). Taken together, these data indicate that parasites lacking either of the biotin ligases can progress through early liver-stage development, but that *ΔHCS1* parasites display a growth phenotype.

Deletion of *Pb*HCS1, but Not *Pb*HCS2, Results in the Loss of Liver-Stage Protein Biotinylation. To assess liver stage biotinylation in the biotin ligase deletion lines, immunofluorescence analysis was performed on HepG2 cells at 48 hpi with WT, *ΔHCS1*, or *ΔHCS2* parasites. *Pb*ACC expression was visualized by using antibodies against the BCCP domain of *Pf*ACC, verifying that the enzyme is expressed normally in both deletion lines (Fig. 6*A*). Although we did not detect any change in biotinylation in *ΔHCS2* parasites, we observed a complete loss of protein biotinylation in *ΔHCS1* parasites (Fig. 6 *B* and *C*). These data indicate that *Pb*HCS1 is essential for protein biotinylation in liver-stage parasites.

Parasites Lacking *Pb*HCS1 Show Delayed Progression Through Liver Stages in Vivo. We measured the prepatent period of the biotin ligase deletion lines by inoculating mice with salivary-gland sporozoites and measuring the length of time before blood-stage parasites were detected microscopically. We found that i.v. injection of 500 sporozoites per mouse did not always result in blood-stage infection, and that this was particularly true for the *ΔHCS1*sporozoites (*SI Appendix*, Table S1). Increasing the inoculum to 5,000 sporozoites per mouse reproducibly produced a blood-stage infection and revealed an average delay in patency of 2.1 d for *ΔHCS1* parasites compared with WT controls. By contrast, *ΔHCS2* parasites were indistinguishable from WT in the number of mice that achieved a patent blood-stage infection and the prepatent period. Thus, deletion of *Pb*HCS1, but not *Pb*HCS2, significantly impairs the ability of malaria parasites to progress from the liver stages to the blood stages.

Deletion of *Pb*HCS1 Affects Late Liver-Stage Development and Reduces Merosome Production. We visualized merozoite morphology by immunofluorescence microscopy at the late liver-stage time point of 56 h. By using antibodies specific for MSP1 (merozoite surface protein 1), we generally observed reduced numbers of merozoites in *ΔHCS1* parasites compared with WT or *ΔHCS2* cells (Fig. 7*A*). Consistent with this observation, the size of the parasitophorous vacuole (assessed by using antibodies specific for the parasitophorous vacuole protein UIS4) appeared to be smaller in *ΔHCS1* parasites (Fig. 7*B*). On average, *ΔHCS1* parasites were approximately 73 μm^2 smaller at the 56-h time point, whereas no significant size decrease was observed in *ΔHCS2* cells (Fig. 7*C*). We next measured the number of merosomes (i.e., membrane-bound clusters of merozoites) released from HepG2 cells infected with WT or *ΔHCS1* parasites at 65 h post invasion. From independent experiments, each performed in triplicate, we found that fewer merosomes were produced by *ΔHCS1* parasites compared with WT controls (Table 1). Taken together, these results show that deletion of *Pb*HCS1 results in late liver-stage defects, and suggests that biotin metabolism is important for this stage of development.

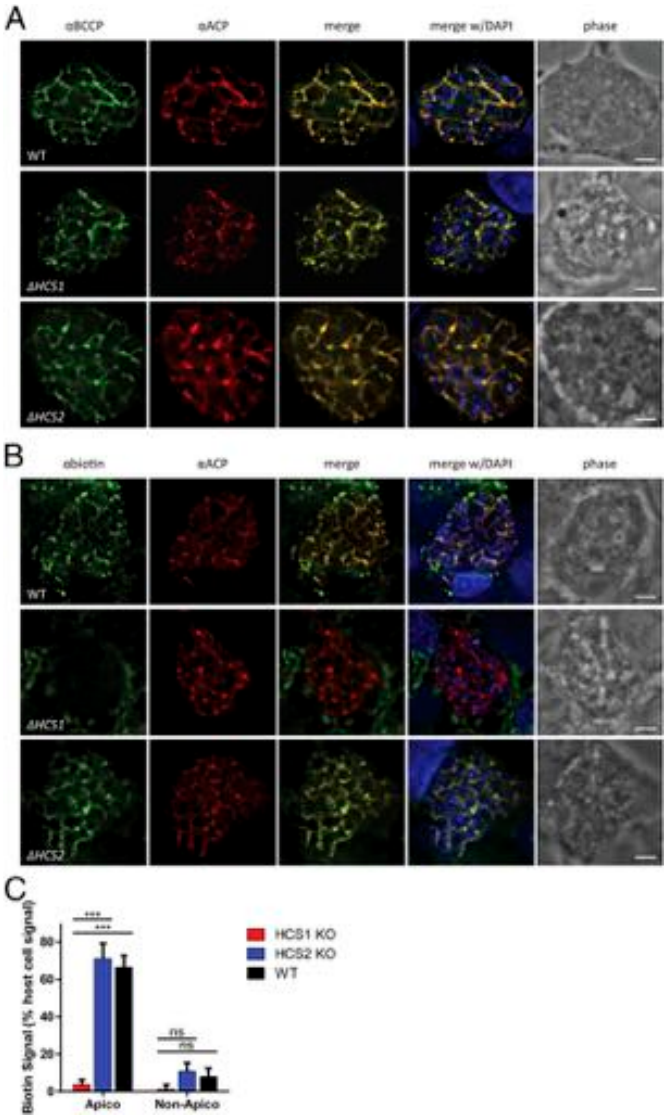

Fig. 6. Deletion of *Pb*HCS1, but not *Pb*HCS2, results in the loss of liver-stage protein biotinylation. (*A*) Immunofluorescence analysis of HepG2 cells at 48 hpi with WT, *ΔHCS1*, or *ΔHCS2 P. berghei* showing that *Pb*ACC (αBCCP) is expressed in the apicoplast (αACP) in both deletion lines. (*B*) Immunofluorescence analysis similar to *A* showing that biotinylated protein (αbiotin) can be detected in the apicoplast (αACP) in WT and *ΔHCS2* parasites, but not *ΔHCS1* parasites. Regions of protein biotinylation surrounding the parasite are presumably host mitochondria, which are thought to be recruited to the parasitophorous vacuole (52). (Scale bars: 5 μm.) (*C*) Biotin signal associated with the apicoplast and nonapicoplast compartments is expressed as a percentage of the total biotin signal found in the host cell. Error bars represent SEM from four or more independent experiments (*ΔHCS1*, $n = 30$, $n = 6$ replicates; *ΔHCS2*, $n = 13$, $n = 4$ replicates; WT, $n = 22$, $n = 6$ replicates). ns, not significant; $***P < 0.001$.

Host Biotin Is Required for Normal Liver-Stage Development. We then tested whether parasites require biotin scavenged from the host to complete liver-stage development. We cultured HepG2 cells by using media that had been dialyzed against avidin to remove biotin (which is present in serum) and then supplemented with additional avidin. Importantly, this biotin-depletion medium did not affect hepatocyte morphology or survival over a 6-d test period (*SI Appendix*, Fig. S11). Cells were seeded and maintained in biotin-depleted media or normal media and infected with

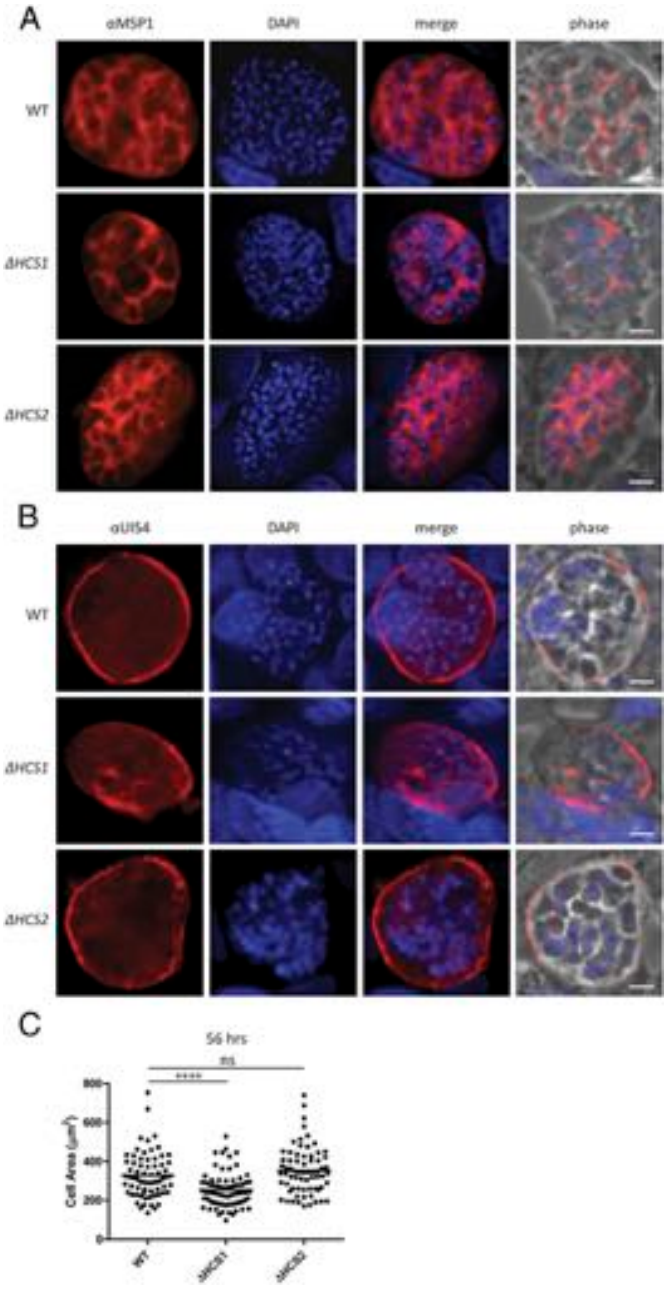

Fig. 7. Liver-stage development is abnormal in *ΔHCS1* parasites. (*A*) Immunofluorescence analysis of infected HepG2 cells 56 hpi with WT, *ΔHCS1*, or *ΔHCS2 P. berghei*. The parasite plasma membrane is illuminated with the late liver-stage marker αMSP1, and the nuclei are labeled with DAPI. (*B*) Immunofluorescence analysis similar to *A* at 56 hpi by using antibodies specific for the parasitophorous vacuole protein UIS4. (*C*) Quantification of cell area at 56 hpi from three biological replicates with at least 20 measurements per parasite strain. (Scale bars: 5 μm.) ns, not significant; ****$P < 0.0001$.

WT or *ΔHCS1*sporozoites, and merosomes were collected and counted 65 h post invasion. At least five replicates were used for each condition. Under normal biotin-replete conditions, we found that there was an approximately fourfold reduction in the number of merosomes produced by *ΔHCS1* parasites compared with WT (Table 1); however, the size of the *ΔHCS1* merosomes appeared consistently smaller than those of WT parasites. We therefore quantified total merozoite numbers by using quantitative PCR and found that *ΔHCS1*merosomes contain fewer merozoites. We observed that the number of merozoites per merosome was ∼10-fold less in the *ΔHCS1* parasites, which, when combined with the lower number of merosomes produced by this mutant, results in a 40-fold overall decrease in the number of merozoites produced. This could account for the significant difference in the prepatency assays (*SI Appendix*, Table S1). Under biotin-depleted conditions, fewer merozoites were produced by WT and *ΔHCS1* parasites than were produced by the mutant when grown in the presence of biotin (Table 1). We assessed the effect of biotin depletion on infected HepG2 cells with immunofluorescence analysis and confirmed that host and parasite biotinylation was significantly reduced (*SI Appendix*, Fig. S12). Together, these data indicate that liver-stage parasites rely on biotin scavenged from the host, and that biotin is required for normal liver-stage development. These data also suggest that perturbation of biotin metabolism in the host cell may further impede the development of liver-stage parasites.

Biotin Is Required for Merosome Infectivity. We assessed the ability of merosomes produced in vitro to initiate blood-stage infections in mice. Merosomes were produced as described earlier in HepG2 cells cultured in biotin-depleted media or normal media. Across three independent experiments, merosomes produced in normal medium were usually infectious (14 of 14 mice for WT and 9 of 13 mice for *ΔHCS1*) with a delay to patency for *ΔHCS1* similar to that observed in *SI Appendix*, Table S1. However, WT merosomes produced under biotin-deficient conditions were infectious in only 1 of 14 mice. Quantification of total merozoites indicates that the reduced infectivity was not caused by lower numbers of merozoites produced (Table 2). Mutant parasites cultured in biotin-deficient medium were never infectious. When combined with the data presented in Table 1, these experiments show that biotin deficiency affects the infectivity of merozoites as well as reducing the total number of merosomes produced.

Discussion

We identified one biotinylation substrate (ACC) in malaria parasites, but two biotin ligases (HCS1 and HCS2). This unusual arrangement seems to be conserved in all species of malaria parasites; however, the organization of biotin metabolism is quite divergent among different apicomplexan genera. The apicomplexan *Cryptosporidium parvum* does not contain an apicoplast and encodes a cytosolic ACC and cytosolic biotin ligase. By contrast, *Theileria annulata* lacks both ACC and biotin ligases. In *T. gondii*, two biotinylated proteins (ACC and pyruvate carboxylase) were

Table 1. Host biotin is required for normal liver stage development

Expt	Line	Biotin	Mrsm per well (SD)	Mzt/Mrsm	Mzt per well ×10³
1	WT	+	6,900 (1,924)	—	—
	ΔHCS1	+	1,550 (788)	—	—
2	WT	+	17,390 (5,663)	3,307	58,000
	WT	–	670 (303)	216	140
	ΔHCS1	+	3,270 (434)	304	990
	ΔHCS1	–	2,330 (1,492)	60	140
3	WT	+	1,500 (418)	4,867	7,300
	WT	–	313 (314)	319	100
	ΔHCS1	+	646 (267)	480	310
	ΔHCS1	–	250 (194)	148	37
4	WT	+	3,000 (576)	3,667	11,000
	WT	–	438 (304)	685	300
	ΔHCS1	+	958 (359)	501	480
	ΔHCS1	–	250 (188)	220	55

Expt, experiment number; Mrsm, merosome; Mzt, merozoite.

Table 2. Biotin is required for merosome infectivity

Expt	Line	Biotin	Mrsm injected	Mice positive	P0, d	Mzt injected×10^3
1	WT	+	1,000	4/4	3.5	—
	WT	–	1,000	0/4	*	—
	Δ*HCS1*	+	1,000	3/3	5.7	—
	Δ*HCS1*	–	1,000	0/1	*	—
2	WT	+	2,500	5/5	3.6	27,000
	WT	–	2,500	0/5	*	520
	Δ*HCS1*	+	2,500	2/5	5.5	170
	Δ*HCS1*	–	2,500	0/5	*	130
3	WT	+	2,000	5/5	5.6	6,700
	WT	–	2,000	1/5	7	350
	Δ*HCS1*	+	2,000	4/5	7.5	320
	Δ*HCS1*	–	2,000	0/5	*	160

Expt, experiment number; Mice positive, fraction of mice infected with blood stage parasites; Mrsm, merosome; Mzt, merozoite; P0, prepatent period.
*Parasites were not detected 1 mo after merosome injection.

identified (29), but there is only one predicted biotin ligase (TGME49_089760).

The duplication of biotin ligases in malaria parasites is similar to the situation found in the plant *A. thaliana*. The two HCS genes in *A. thaliana* share 71% sequence identity and likely resulted from a gene duplication event. Similarly, HCS1 and HCS2 from *P. falciparum* share 47% sequence identity and are likely paralogs created by gene duplication. *At*HCS1 is essential for viability, whereas disruption of the *At*HCS2 gene does not lead to any observable phenotype (21). Similarly, deletion of *P. falciparum* HCS1 results in a growth defect whereas deletion of HCS2 does not (*SI Appendix*, Table S1). It is possible that HCS2 and *At*HCS2 share a common role that has yet to be determined.

Both biotin ligase paralogs are predominantly located in the cytosol in the blood stages of *P. berghei* (Fig. 2*C*) and *P. falciparum* (Fig. 3 *A* and *B*). It was anticipated that at least one of the ligases would be located in the apicoplast because this subcellular compartment was thought to contain ACC (30, 31). Indeed, ACC is located in the apicoplast in *P. berghei* and *P. falciparum* (Fig. 1 *C* and *E*), consistent with apicoplast localization of ACC in the related apicomplexan parasite *T. gondii* (29). Although ACC is biotinylated in *T. gondii* (29), it is not biotinylated in blood-stage malaria parasites (Fig. 1 *D* and *F*). We found this result to be surprising and used a genetic probe to determine whether any biotin ligase activity is located in the apicoplast. When we expressed the BCCP domain of ACC in blood-stage *P. falciparum* parasites, we found that the probe was biotinylated when expressed in the cytosol, but that there was no detectable biotinylation when it was expressed in the apicoplast (Fig. 4). This result is consistent with the cytosolic localization of both biotin ligases and indicates that the apicoplast of blood-stage parasites does not contain any enzymes with biotin ligase activity. Taken together, these results show that blood-stage malaria parasites express ACC and two active biotin ligases, but ACC is not biotinylated because all biotin ligase activity is confined to the cytosol whereas ACC is exclusively located in the apicoplast.

The fact that ACC is not biotinylated in blood-stage malaria parasites suggests that biotin is not needed during this stage of parasite development. We cultured *P. falciparum* parasites expressing the BCCP domain in biotin-depleted growth medium. These parasite lines grew equally well regardless of whether biotin was added back to the medium (Fig. 5*A*). Subsequent analysis of parasites expressing BCCP in the cytosol demonstrated that BCCP was biotinylated only when biotin was added back to the depleted medium (Fig. 5*B*). These experiments show that blood-stage parasites do not synthesize biotin and do not rely on this vitamin for growth, but can import external biotin when it is supplied in the medium. Even when parasites were cultured in growth medium designed to increase the reliance on fatty acid metabolism, there was still no growth phenotype associated with biotin depletion (Fig. 5*C*).

The ability of blood-stage malaria parasites to grow without biotin has interesting consequences for fatty acid metabolism. All known FAS and ELO pathways use malonyl-CoA for chain elongation. ACC is the only predicted source of malonyl-CoA in malaria parasites, and thus should be required for FASII and ELO activity. Key enzymes of the FASII pathway, such as FabI, have been knocked out in human and rodent malaria species, demonstrating that FASII is dispensable for blood-stage replication (13, 14). The lack of ACC biotinylation in blood-stage parasites further suggests that FASII cannot function during this stage of parasite development. Fatty acid elongation, however, can still be observed in *ΔFabI* parasites, implying that the ELO pathway is active in the blood stages of *P. falciparum* and *P. berghei* (14). We used the *P. falciparum ΔFabI* line to show that radiolabeled acetate is still incorporated into parasite fatty acids regardless of whether biotin is present in the growth medium, and, furthermore, the incorporation of acetate was the same between *ΔFabI* parasites and the parental line (Fig. 5*D*). Taken together, these data suggest that FASII is inactive in blood-stage *P. falciparum* parasites whereas these parasites have ELO activity that does not depend on biotin or ACC. The ELO pathway could rely on another source of malonyl-CoA, such as a malonyl-CoA synthetase (32).

In liver-stage malaria parasites, the arrangement of ACC and the biotin ligases appears to be the same as it is in blood stages. The ACC is located in the apicoplast (Fig. 1) whereas the biotin ligases are predominantly located in the cytosol (Fig. 2 and *SI Appendix*, Fig. S3). The obvious difference is that apicoplast proteins (presumably ACC, although there may be others) are biotinylated, and this raised the question of which ligase is responsible for this activity. To answer this question, we deleted the genes encoding HCS1 and HCS2 in *P. berghei* and found that HCS1 is exclusively responsible for protein biotinylation whereas deletion of HCS2 has no detectable liver-stage phenotype (Fig. 6). This result suggests that HCS1 is partially located in the apicoplast of liver-stage parasites, albeit at low levels compared with the majority of HCS1 located in the cytosol (Fig. 2*A*). There are examples in other organisms of dual targeting of biotin ligases to different cellular compartments, which is regulated by alternative splicing of a single mRNA or alternative translation initiation sites. This has been shown in some detail in *A. thaliana* (21) and has been suggested in *Pisum sativum* (20) as well as humans (33). In addition, there are examples of dual targeting of proteins to the apicoplast, mitochondrion, and cytosol in malaria parasites (34–36), although the mechanisms are not well understood. One suggested mechanism is the use of an ambiguous targeting sequence that is inefficiently recognized by the protein sorting machinery, so that a fraction of the total protein is imported into an organelle and the remainder is retained in the cytosol (37). Regardless of the mechanisms involved, our results show that biotinylation by HCS1 is stage-specific, occurring in liver-stage but not blood-stage malaria parasites.

P. berghei parasites lacking HCS1 have a defect in liver-stage development that begins early in parasite development (*SI Appendix*, Fig. S10) and becomes more obvious by 56 h post invasion (Fig. 7). *ΔHCS1* parasites produce fewer merosomes than WT parasites, and these merosomes contain significantly fewer merozoites (Table 1). In general, late liver-stage developmental defects and severely curtailed formation of merosomes are hallmarks of KO parasites with FASII gene deletions (13, 14) or deletions of genes required for the FASII pathway (15, 38). Deletion of FASII-related genes in *P. berghei* [*ΔFabI* (14) and *ΔLipB* (38)] results in a 4-d delay to patency, with few parasites able to complete liver-stage development and almost no merosome formation. By contrast, *ΔHCS1* parasites display a 2.1-d delay to patency and

quantifiable merosomes. If HCS1 is responsible for activating ACC to supply malonyl-CoA for FASII, why is the *ΔHCS1* phenotype different from that of *ΔFabI* or *ΔLipB*? One explanation for the less severe *ΔHCS1* phenotype is that the parasites have another source of malonyl-CoA, allowing them to partially bypass the need for HCS1. Hepatocyte ACC produces malonyl-CoA, and it is possible that this metabolite can be scavenged. This explanation is consistent with the observation that WT and *ΔHCS1* parasites produce fewer merozoites when cultured in biotin-depleted medium (Table 1). Presumably, the deletion of HCS1 affects only parasite ACC activity, whereas biotin depletion affects parasite and host cell ACC activity.

To assess the combined effects of host cell and parasite biotin deprivation, we conducted merosome infection experiments. We found that WT and mutant *ΔHCS1*merosomes produced in vitro by host cells cultured in complete medium were usually infectious in mice, but were almost never infectious if they were produced in biotin-deficient conditions (Table 2). The dramatic decrease in infectivity could result from the merozoites failing to egress from the membrane-bound merosomes, or it could be that the merozoites are defective. It is difficult to distinguish between these possibilities in our experiments; however, the fact that many merozoites are released during the injection process suggests that the merozoites themselves are less infectious.

These results raise the possibility that biotin is a host nutrient that could affect parasite development, as has been suggested for other nutrients (39). In humans, biotin deficiency is almost invariably caused by mutations in an essential enzyme called biotinidase, which is required to make use of biotin obtained from dietary sources (40). More than 165 biotinidase mutations have been described in humans (41). In some cases, these mutations are severe enough to cause disease, but it is more common for these mutations to decrease biotin utilization without causing disease. Although the data currently available are not sufficient to implicate biotin deficiency in protection from malaria infection, there is some evidence that biotinidase mutations are more prevalent in certain African populations (42).

In conclusion, we found that ACC is expressed in the apicoplast organelle in liver- and blood-stage malaria parasites, but it is biotinylated only in the liver stages, suggesting that ACC activity is regulated through stage-specific biotinylation. Because ACC is the first committed step in FASII pathways, protein biotinylation may serve as a molecular switch controlling fatty acid biosynthesis in malaria parasites. Gene deletion experiments showed that the biotin ligase HCS1 is solely responsible for biotinylation in liver-stage parasites. Parasites lacking HCS1 show a late liver-stage defect and produce fewer merosomes with abnormally low numbers of merozoites per merosome. Biotin depletion results in more severe developmental defects, resulting in merozoites that are no longer capable of blood-stage infection. These results imply that host and parasite biotin metabolism are required for normal liver-stage progression and merozoite infectivity. Thus, the development of liver-stage malaria parasites may be linked to the nutritional status of the host, as neither the parasite nor the human host can synthesize biotin.

Materials and Methods

Cells and Antibodies. HepG2 human liver carcinoma cells [HB-8065; AmericanType Culture Collection (ATCC)] were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FCS, 2 mM glutamine, and 1× penicillin–streptomycin. The following antibodies were used: mouse monoclonal α-biotin (no. 200–002-211; Jackson) at 1:250 or 1:500 dilution; rabbit BCCP antiserum (10) (raised against the *P. falciparum* antigen but recognizes *P. falciparum* and *P. berghei* ACC) at 1:100 (*Pf*) or 1:250 (*Pb*) dilution; rabbit or rat polyclonal αACP (43, 44) (raised against the *P. falciparum* antigen but recognizes *P. falciparum* and *P. berghei* ACP) at 1:500 (*Pf*) or 1:1,000 (*Pb*) dilution; mouse monoclonal αCSP 3D11 (27) at 1:1,000 dilution; rabbit αUIS4 (28) at 1:1,000 dilution; *P. yoelii* MSP1-19 rabbit antiserum (MRA-23, MR4; ATCC) at 1:1,000 dilution; and mouse monoclonal αGFP (Roche) at 1:200 (*Pf*) or 1:1,000 (*Pb*) dilution. The corresponding secondary antibodies conjugated to Alexa Fluor-488 or Alexa Fluor-594 (Invitrogen) were used as indicated.

Immunofluorescence Analysis. To visualize liver-stage parasites, HepG2 cells were seeded at a density of 10^5 cells per well on Permanox eight-well chamber slides coated with collagen (no. 354236; BD Biosciences). *P. berghei* ANKA sporozoites were harvested from infected *Anopheles stephensi* mosquitoes 18–24 d post blood meal, quantified, and centrifuged onto the HepG2 cells at a density of 2×10^4 sporozoites per well. At the time point indicated, cells were fixed with 4% paraformaldehyde for 20 min at room temperature, washed with PBS solution, and permeabilized with methanol overnight at −20 °C. Slides were blocked and probed in 1% BSA in PBS solution and mounted with Prolong Gold Antifade Reagent with DAPI (Invitrogen). Average cell area was quantified by using Volocity software (PerkinElmer). Immunofluorescence analysis of *P. berghei* and *P. falciparum* blood-stage parasites was carried out as previously described (45) with a few modifications (46). The *SI Appendix* provides more details.

***P. falciparum* Transfections.** Parasite transfections were carried out by using the mycobacteriophage Bxb1 integrase system developed by Nkrumah et al. (47) and modified by Spalding et al. (48). Vector construction using the primers listed in *SI Appendix*, Table S2 is described in the *SI Appendix*.

Endogenous Tagging and Localization of *P. berghei* Biotin Ligases. Double cross-over replacement constructs were made for the *P. berghei* biotin ligase genes *HCS1* (PBANKA_051100) and *HCS2* (PBANKA_132360). *P. berghei* ANKA genomic DNA was amplified and cloned into a vector designed to append a 3xHA tag to the 3′ end of the ligase genes. A single cross-over strategy was used to generate GFP-tagged parasite lines (*SI Appendix*). Constructs were linearized and transfected into *P. berghei* ANKA according to Janse et al.(49).

Biotin Ligase Activity Assays and Affinity/Western Blot Analysis. The biotin ligase genes were subcloned into expression vector pMALcHT (50) for expression and purification as MBP fusion proteins. The truncated BCCP domain of *Pf*ACC was expressed as a GST fusion protein in BL21-Star(DE3) cells by using a modified pGEX-4T-3 expression vector (pGEXT) and purified as previously described (10). The biotin ligase in vitro assay was carried out by using pure recombinant BCCP and biotin ligase proteins in the presence of ATP and biotin. Biotinylated BCCP was detected by affinity blot using streptavidin coupled to peroxidase (ultrasensitive; Sigma), and the presence of BCCP protein was confirmed by anti-BCCP Western blot. For the cell-free assay, *P. falciparum* parasites were isolated by saponin lysis followed by sonication to release parasite proteins. BCCP, biotin, and ATP were added to the parasite lysates, and biotinylation activity was assessed by affinity and Western blot.

Growth Assay and Radiolabel Incorporation. Biotin-depleted medium was prepared using RPMI medium 1640 modified with L-glutamine without phenol red and biotin (R9002-01; US Biological), which was supplemented with 25 mM Hepes, 0.375% sodium bicarbonate, 12.5 μg/mL hypoxanthine, 5 g/L AlbuMAX II, and 25 μg/mL gentamicin. For each parasite line, the cultures and red blood cells were washed twice in 10 mL biotin-free RPMI to remove biotin and then divided into parallel cultures for growth assays conducted with or without biotin supplementation. Lipid-free BSA (Sigma) was reconstituted with palmitic acid (Sigma) and potassium oleate (Fluka) and used in lieu of AlbuMAX for growth assays under minimal lipid conditions. For radioactive biotin incorporation experiments, *P. falciparum* cultures were maintained with daily feedings of biotin-free medium. Parasite cultures were labeled for 3 d with 0.48 nmol ^{3}H-biotin (American Radiolabeled Chemicals) or 29.2 nmol of freshly prepared ^{35}S-biotin (10) each day for 3 d. Label incorporation was assessed by autoradiography. For acetate incorporation, synchronized ring-stage parasites were incubated for 24 h with 10 μCi/mL ^{14}C-acetate, after which parasites were isolated by saponin lysis. Fatty acid methylesters were extracted and resolved by TLC as described in *SI Appendix*.

***P. berghei* Biotin Ligase KOs.** To generate the double cross-over KO construct for *PbHCS1* and *PbHCS2*, homology arms composed of the 5′ and 3′ UTR of *PbHCS1* and *PbHCS2* were cloned into the pDEF-hDHFR plasmid (51). Constructs were linearized and transfected into *P. berghei* as described by Janse et al. (49) with a few modifications (*SI Appendix*).

***P. berghei* Liver-Stage Development and Infectivity Assays.** For prepatency assays, salivary gland sporozoites were harvested from infected *A. stephensi* mosquitoes and injected into mice i.v. followed by daily monitoring of

parasitemia by blood smear. Merosome production was assessed by collecting the supernatant from HepG2 cells infected with salivary gland sporozoites at 65 hpi. Merosomes were quantified in a hemocytometer, and the DNA content of the merosome samples was determined with quantitative real-time PCR. For infectivity assays, harvested merosomes were quantified and prepared for tail-vein injection into mice. Blood-stage parasitemia was subsequently monitored by microscopy.

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7.1.1 Tables

Table S1. *PbΔHCS1* sporozoites exhibit decreased infectivity *in vivo*

	Mouse strain	Parasite line	Sporozoites injected	#mice positive #mice injected	Prepatent period (days)	Growth delay (days)
(Δ*HCS1* clone 1)	Swiss Webster	WT	500	5/5	4.8	
	Swiss Webster	WT	5000	5/5	3.6	
	Swiss Webster	Δ*HCS1*	500	2/5	5.5	0.7
	Swiss Webster	Δ*HCS1*	5000	5/5	6.0	2.4
(Δ*HCS1* clone 2)	Swiss Webster	WT	500	5/5	5.0	
	Swiss Webster	WT	5000	5/5	4.0	
	Swiss Webster	Δ*HCS1*	500	3/5	7.3	2.3
	Swiss Webster	Δ*HCS1*	5000	5/5	6.2	2.2
(Δ*HCS1* clone 3)	Swiss Webster	WT	5000	4/5	4.5	
	Swiss Webster	Δ*HCS1*	5000	4/5	6.8	2.3
	Swiss Webster	WT	5000	5/5	4.2	
	Swiss Webster	Δ*HCS1*	5000	4/5	6	1.8
						2.1 (0.26)*
(Δ*HCS2* clone 1)	Swiss Webster	WT	500	4/5	3.8	
	Swiss Webster	WT	5000	5/5	3.6	
	Swiss Webster	Δ*HCS2*	500	5/5	3.8	0.0
	Swiss Webster	Δ*HCS2*	5000	5/5	3.4	-0.2
(Δ*HCS2* clone 2)	Swiss Webster	WT	500	4/5	5.8	
	Swiss Webster	WT	5000	5/5	3.8	
	Swiss Webster	Δ*HCS2*	500	1/5	5.0	-0.8
	Swiss Webster	Δ*HCS2*	5000	5/5	3.8	0.0
						0.0 (0.20)*

* Average (standard deviation) from six experiments with Δ*HCS1* (from two independent transfections) or triplicate experiments with ΔHCS2 using the 5000 sporozoite inoculum.

Table 7.1: Supplementary Table 1

7.1.2 Figures

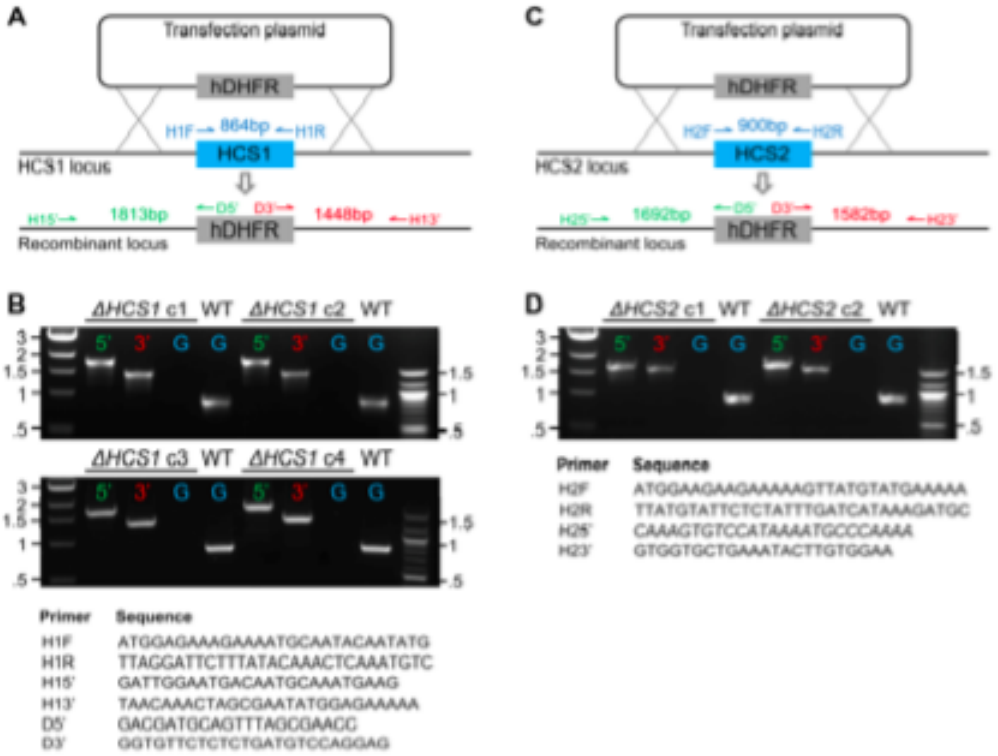

Figure S8. Generation of *ΔHCS1* and *ΔHCS2 P. berghei*. **A)** The gene encoding *Pb*HCS1 was knocked out through double homologous recombination. The knockout constructs were designed to excise the entire open reading frame of the gene and replace it with a hDHFR resistance cassette. Primer pairs and amplicon sizes are shown for primers designed to detect the HCS1 gene (blue), 5' integration (green), and 3' integration (red). **B)** Correct integration of the hDHFR cassette and loss of the *HCS1* gene was verified in four clones from two independent transformations by diagnostic PCR using the primers described in (A). PCR reactions with gene-specific primers (blue G) detect *HCS1* in wild type parasites (WT), but not in either knockout clone. **C)** Primer pairs and amplicon sizes are shown in conjunction with the strategy to knockout the gene encoding *Pb*HCS2. **D)** The correct genotypes of two independent *HCS2* knockout clones were verified by diagnostic PCR as in (B).

Figure 7.1: Supplementary Figure 8

7.2 Curriculum vitae

Hugo Jhun
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Education

Johns Hopkins Bloomberg School of Public Health, Baltimore, MD
Ph.D.: Molecular Microbiology and Immunology; Fall 2011- February 2018
Thesis: "Probing the necessity of mitochondrial lipoylation in *Plasmodium*"
Relevant coursework in immunology, microbiology, biostatistics, virology, vaccinology and epidemiology

Queens College of the City University of New York, Flushing, NY
B.A.: Biochemistry; 2009-2011
Thesis Project: Synthesized substrates to assay human Nek2 kinase activity against an anti-cancer compound library using radiometric and spectroscopic methods

Queens College of the City University of New York, Flushing, NY
M.A.: English Literature; 2005-2009
Thesis: "Disparate Housewives: Marriage in the 19th Century as seen through *Pride and Prejudice* and *The Heavenly Twins*"

Honors and Awards

Johns Hopkins Bloomberg School of Public Health
Sommer Scholarship – competitive schoolwide fellowship, providing leadership and team-building training for students focused on public health
Delta Omega Scholarship – annual competitive scholarship for projects of merit awarded by the Alpha Chapter of the Delta Omega Honorary Public Health Society
Frances Coventry Scholarship – departmental fellowship named after alumna

Queens College of the City University of New York, Flushing, NY
Stanley G. Konkol Memorial Award, Departmental Service Award, Departmental Honors

Scientific Skills

Graduate Thesis Work

- Developed a wide array of techniques in the human and mouse models of malaria, including molecular cloning, genetics (including CRISPR/Cas9), transfection of various cell types, Western blotting, protein purification, radiolabeling, immunofluorescence microscopy, yeast-two-hybrid assays, plasmid shuffle, immunoprecipitation, cell culture, mouse work, high throughput screening, *E.coli*/yeast work, and basic flow cytometry
- Reviewed, edited and evaluated scientific manuscripts, grant applications and fellowship applications
- Trained, assisted and advised multiple students on various projects, including high-throughput drug screens, enzymology and novel knockout/knockdown technologies

Co-organizer of 2016 Malaria Symposium

- Recruited by Nobel Laureate Dr. Peter Agre, director of the Johns Hopkins Malaria Research Institute
- Worked with fellow scientists in diverse specialties and administrators to set up the international meeting
- Recruited two leaders in the malaria field to give keynote talks in drug development and epidemiology
- Organized and evaluated scientific submissions presentation, and helped facilitate review criteria and review panels
- Meeting participants included leaders from academia, government, the military and industry

Undergraduate Work

- Employed peptide/organic/inorganic chemistry to synthesize substrates for enzymatic assay against NCI compound libraries
- Determined product purity using HPLC and mass-spectrometry
- Assayed activity using radiometry and UV-Vis spectroscopy
- Purified recombinant protein using affinity chromatography
- Trained in NMR, gas chromatography and various spectroscopic techniques

Previous Work Experience

English Teacher, Forest Hills High School (2001-2007)

Certified NYC Public School Teacher for English
Taught all levels (9^{th}-12^{th} grade), including remedial, ESL, honors and drama classes

Leadership and Service

Johns Hopkins Bloomberg School of Public Health, Baltimore, MD

- Organized a monthly journal club dedicated to exploring various topics in biology, microbiology and immunology
- Served as a student representative for external review boards for the Department of Molecular Microbiology and Immunology and the Johns Hopkins Malaria Research Institute to provide evaluations for both organizations
- Founder and President of the Lab Science Student Group (2014-2015) designed to improve student relationships between lab departments and provide professional development opportunities
- Facilitated the Tropical Medicine Dinner Club, a semi-monthly meeting and seminar of DC/Baltimore scientist working on tropical medicine, as student liaison for logistics and planning

Faculty Member at Forest Hills High School, Forest Hills, NY

- Established the English Honor Society to support highly performing students with extracurricular activities
- Co-headed senior thesis committee to establish a rigorous, research-based graduation requirement
- Served as member of curriculum development committee to review and overhaul the curriculum
- Volunteered to teach pilot program targeted to poorly performing students with behavioral issues
- Trained two student teachers in lesson planning, student evaluation and classroom management

Publications, Posters and Presentations

Jhun, H., Spalding, M., Sinnis, P., Prigge S. "Probing mitochondrial lipoylation in *Plasmodium*" (*preparing for submission*)

Poster presentations
- 2018 Delta Omega Poster Competition, Johns Hopkins University, MD
- 2015 American Society for Biochemistry and Molecular Biology, Boston, MA
- 2014 Molecular Parasitology Meeting, Woods Hole, MA.

Oral Presentations
- 2014 Malaria Research Institute Seminar, Johns Hopkins University, MD
- 2016 Tropical Medicine Dinner Club, Johns Hopkins University, MD

Jhun, H., Spalding, M.S., Prigge, S.T. "Mitochondrial lipoylation is essential in *P. falciparum*" *mBio* (*submitted*)

Dellibovi-Ragheb T.A., **Jhun H.**, Goodman, C.D., Walters, M.S., Ragheb, D.R.T., Matthews, K.A., Rajaram, K., Mishra, S., McFadden, G.I., Sinnis, P., Prigge, S.T. Host Biotin is Required for Liver Stage Development in Malaria Parasites. *PNAS* (2018)

Das, T.K., Dana, D., Paroly, S.S., Perumal, S.K., Singh, S., **Jhun, H**., Pendse, J., Cagan, R.L., Talele, T.T., Kumar, S. Centrosomal kinase Nek2 cooperates with oncogenic pathways to promote metastasis. *Oncogenesis*. (2013)

Lamb, H.M., **Jhun, H**., and Hardwick J.M. "Bax and Bcl-xL cooperate to alter curvature of cellular membranes" Poster presentation at the 2012 Keystone Symposia: Mitochondrial Dynamics and Function, Banff, Canada.